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Two New Antioxidant Malonated Caffeoylquinic Acid Isomers in Fruits of Wild Eggplant Relatives

Chunhui Ma,[†] Keyvan Dastmalchi,[†] Bruce D. Whitaker,[*,‡] and Edward J. Kennelly[†]

[†]Department of Biological Sciences, Lehman College and The Graduate Center, The City University of New York, 250 Bedford Park Boulevard West, Bronx, New York 10468, United States

[‡]Food Quality Laboratory, Building 002, Room 117, Beltsville Agricultural Research Center—West, Agricultural Research Service, U.S. Department of Agriculture, 10300 Baltimore Avenue, Beltsville, Maryland 20705, United States

ABSTRACT: Fruits of the cultivated eggplant species *Solanum melongena* and its wild relative *Solanum incanum* have a high content of hydroxycinnamic acid conjugates, which are implicated in the human health benefits of various fruits and vegetables. Monocaffeoylquinic acid esters, in particular 5-*O*-(*E*)-caffeoylquinic acid, are usually predominant in solanaceous fruits and tubers. Two closely related caffeoylquinic acid derivatives with longer C_{18} HPLC retention times than those of monocaffeoylquinic acids are minor constituents in cultivated eggplant fruit. In a prior study, the two compounds were tentatively identified as 3-*O*-acetyl- and 4-*O*-acetyl-5-*O*-(*E*)-caffeoylquinic acids and composed ≤2% of the total hydroxycinnamic acid conjugates in fruit of most *S. melongena* accessions. It was recently found that the pair of these caffeoylquinic acid derivatives can compose 15−25% of the total hydroxycinnamic acid conjugates in fruits of *S. incanum* and wild *S. melongena*. This facilitated C_{18} HPLC isolation and structural elucidation using ^{1}H and ^{13}C NMR techniques and HR-ToF-MS. The isomeric compounds were identified as 3-*O*-malonyl-5-*O*-(*E*)-caffeoylquinic acid (isomer 1) and 4-*O*-(*E*)-caffeoyl-5-*O*-malonylquinic acid (isomer 2). Both exhibited free radical scavenging activity, albeit about 4-fold lower than that of the flavonol quercetin dihydrate. By contrast, the iron chelation activities of isomers 1 and 2, respectively, were about 3- and 6-fold greater than that of quercetin dihydrate. Reports of malonylhydroxycinnamoylquinic acids are rare, and only a few of these compounds have been structurally elucidated using both NMR and MS techniques. To the authors' knowledge, these two malonylcaffeoylquinic acid isomers have not previously been reported.

KEYWORDS: wild eggplant relative, *Solanum incanum*, *Solanum melongena*, fruit, malonylcaffeoylquinic acid, antioxidant activity, iron chelation

INTRODUCTION

Phenolic phytochemicals in fruits and vegetables are thought to play a major role in the health-promoting benefits associated with the consumption of these plant products.[1−3] Phenolic acids and other classes of phenylpropanoids are typically strong antioxidants and effective free radical scavengers.[2,4] Consequently, the water-soluble antioxidant activity of fruit and vegetable extracts is often closely correlated with the levels of various phenylpropanoids,[4,5] although other compounds such as ascorbic acid and glutathione contribute as well. A study using four different assays to evaluate the antioxidant activity in 120 vegetables ranked eggplant among the top 10 for scavenging of superoxide.[6] This is attributed to phenolic constituents,[6] including nasunin and related anthocyanins in the skin of pigmented varieties,[7] and a high content of hydroxycinnamic acid conjugates in the flesh.[8,9] Phenolic compounds extracted from eggplant fruit and administered orally to normal and cholesterol-fed rats had a significant hypolipidemic effect.[10] In addition, eggplant extracts were found to inhibit protein-activated receptor 2 inflammation associated with atherosclerosis.[11]

Formerly, it was hypothesized that antioxidant activity of dietary phenolic phytochemicals is directly linked with their protective effects against diseases involving free radical mediated lipid peroxidation and chronic inflammation.[1,2] Recent research, however, has indicated that other, systemic modes of action are likely of greater importance, particularly when the low-level absorption and metabolism of dietary phenolics are considered.[1,2] Two such modes of action supported by emerging evidence are modulation of cell signaling cascades that regulate vital functions (e.g., growth, proliferation, and apoptosis) and activation of endogenous antioxidant defenses.[1−3]

The human health benefits of hydroxycinnamoyl esters of quinic acid, collectively referred to as chlorogenic acids, are attributed to a broad range of biological activities, including free radical scavenging and anti-inflammatory, antiviral, antimicrobial, antimutagenic, and anticarcinogenic action.[12−14] A considerable body of research has focused on the bioactivities of 5-*O*(*E*)-caffeoylquinic acid, the most widely distributed chlorogenic acid, as well as its isomers and derivatives. Recent reports have described in vivo and in vitro antihepatitis B activity,[13] antimicrobial activity against Gram-positive human pathogenic bacteria,[14] reduction of oxidative stress and inflammation in a rat model of inflammatory bowel disease,[15] and in vitro inhibition of human hepatic glucose-6-phosphatase activity associated with prevention of type 2 diabetes.[16] These and other studies have shown that efficacy or potency of individual caffeoylquinic acids is very much dependent on their structure, that is, which hydroxyls on quinic acid are esterified with caffeic acid or linked with other moieties. For example, 4-*O*(*E*),5-*O*(*E*)-dicaffeoylquinic acid was

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a stronger inhibitor of the efflux pump of Gram-positive pathogenic bacteria than 5-*O*(*E*)-caffeoylquinic acid, whereas neither 3-*O*(*E*)-caffeoylquinic acid nor 3-*O*(*E*),5-*O*(*E*)-dicaffeoylquinic acid was inhibitory.[14] Such findings of structure−function relationships have sparked considerable interest in identifying novel, biologically active chlorogenic acid derivatives in crop species and their wild relatives, with the aims of nutraceutical enrichment to yield new functional foods and development of new pharmaceuticals.

Fruits of the cultivated eggplant species *Solanum melongena*, as well as related cultivated and wild species, usually include mainly monocaffeoylquinic acid esters, with 5-*O*(*E*)-caffeoylquinic acid composing up to 95% of the total hydroxycinnamic acid conjugates.[8] However, in extracts of the fruits from two purported *Solanum anguivi* accessions in the USDA eggplant germplasm collection, PI 179745 and PI 183357,[17] we noted that about 15−25% of the 5-*O*(*E*)-caffeoylquinic acid was replaced by a pair of compounds that on C_{18} HPLC eluted later than mono- but earlier than dicaffeoylquinic acids. On the basis of limited ES^- MS and ^1H NMR data, the compounds were previously misidentified as 3-*O*-acetyl- and 4-*O*-acetyl-5-*O*(*E*)-caffeoylquinic acids.[9] In the present study, the two caffeoylquinic acid derivatives were isolated from fruits of accessions PI 179745 and PI 183357 and structurally elucidated using various ^1H and ^{13}C NMR and HR-ESI-MS techniques. Antioxidant and iron-chelating activities of the two compounds relative to those of the flavonol standard quercetin dihydrate were also determined.

MATERIALS AND METHODS

Plant Material and Cultural Methods. Seeds of accessions PI 179745 and PI 183357 were obtained from the USDA, ARS, Plant Genetic Resources Conservation Unit, in Griffin, GA. Although both accessions are designated *S. anguivi* in the USDA-ARS *GRIN* database,[17] seeds from each yielded a mixed population exhibiting phenotypes inconsistent with *S. anguivi*. Two prominent types, one from each accession, showed a short bushy growth habit (0.6 m tall), abundant spines, purple flowers borne singly, and small, round, light-green fruit (2−3 cm diameter) with dark-green stripes and thorny calyces. On the basis of AFLP and morphological data, Furini and Wunder[18] classified both PI 179745 and PI 183357 as *S. incanum* accessions. After examining photos of whole plants, leaves, shoots, flowers, and fruits, two experts in *Solanum* taxonomy, Dr. Michael Nee at the New York Botanical Garden and Dr. Maria Vorontsova at Kew Royal Botanic Gardens, confirmed that PI 183357 is *S. incanum* but agreed that PI 179745 is likely a wild Asian accession of *S. melongena*.

Seedlings were raised in a greenhouse and then transplanted to and grown in a field plot at the Beltsville Agricultural Research Center, Beltsville, MD, using standard horticultural practices for eggplant production in Maryland[19] as previously described.[8]

Four fruits from each of four plants were harvested from about 40 to 45 days postanthesis, when they had reached full size but the seeds had not yet fully developed. The small, round fruit ranged from about 2.0 to 3.0 cm in diameter and from 5.2 to 7.6 g fresh weight. After excision of the peduncle and calyx, the fruits were washed with tap water and blotted dry. They were then quickly diced, and the tissues were frozen in liquid N_2 and lyophilized. For each accession, the pooled freeze-dried tissue from all 16 fruit was pulverized, sieved to remove seeds, and stored as a single sample in a small ziplock bag at −80 °C until used.

Extraction of Hydroxycinnamic Acid Conjugates from Fruit Tissues. Total phenolics were extracted from four 3.0 g samples of the lyophilized, powdered fruit tissue from each of the two accessions by sonicating for 15 min in 30 mL of methanol containing 0.5% butylated hydroxytoluene (BHT). The first methanol extract was decanted after centrifugation and the tissue sample extracted a second time with 30 mL of methanol plus BHT. The two extracts were combined, vacuum filtered through a glass fiber disk in a sintered glass funnel, and then reduced to about 30 mL under a stream of N_2 while heated at 40 °C. An equal volume of 0.1% (10 mM) aqueous phosphoric acid was added, followed by vortexing for 20 s and cooling on ice for 15 min to precipitate the BHT. Extracts were then centrifuged for 3 min at 2000*g* to pellet the precipitate, decanted, and vacuum filtered as before. The filtered supernatants (4 × 60 mL) were each extracted twice with 10 mL of hexane to remove pigments, lipids, and residual BHT. They were then reduced to 20 mL volume under a stream of N_2 while heated at 40 °C prior to extraction three times with 20 mL of ethyl acetate. The four combined ethyl acetate extracts (60 mL each) including >90% of the total hydroxycinnamic acid conjugates were individually N_2 evaporated. The residue from each was dissolved in 4 mL of methanol, which was transferred to a 6 mL amber vial. The vials were flushed with N_2, sealed with a Teflon-lined screw cap, and stored at −80 °C until the extracts were fractionated.

Standards and Reagents. Ethylenediaminetetraacetic acid disodium salt, 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium peroxosulfate, trichloroacetic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), chlorogenic acid, and quercetin dihydrate were purchased from Sigma-Aldrich (St. Louis, MO). Diammonium 2,-2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was from TCI-Ace (Tokyo, Japan). Ultrapure water was prepared using a Milli-RO 12 plus system (Millipore Corp., Bedford, MA).

Isolation of Caffeoylquinic Acid Derivatives 1 and 2 by SPE and HPLC. Aliquots of the fruit tissue hydroxycinnamic acid conjugate extracts from PI 179745 and PI 183357 were analyzed by C_{18} HPLC-DAD as described in ref 9. Together, unknown compounds **1** and **2** composed 16.7 and 24.6% of the total hydroxycinnamic acid conjugates in fruit of PI 179745 and PI 183357, respectively. The extracts were then fractionated on 500 mg Strata-X polymeric solid phase extraction (SPE) tubes (Phenomenex, Torrence, CA). First, methanol was evaporated with a stream of N_2 while samples were held at 40 °C, and the residue was dissolved in 10 mL of deionized water/methanol, 19:1 (v/v). After the Strata-X bed had been washed with 10 mL of methanol and 10 mL of water, the sample was loaded and passed through the sorbent dropwise under gentle N_2 pressure. The tube was then eluted with successive 10 mL volumes of 25, 40, and 60% aqueous methanol, collected as separate fractions. Examination of aliquots of the fractions by C_{18} HPLC-DAD showed that the 25% methanol eluate was enriched in 5-*O*(*E*)-caffeoylquinic acid, whereas the 40% fraction included a higher proportion of the two unknowns and largely excluded dicaffeoylquinic acids. Solvent was N_2 evaporated from the 40% methanol fractions at 40 °C, reducing the volume to 4 mL. These largely aqueous samples were then adsorbed to 200 mg Strata X SPE tubes, followed by a rinse with 4 mL of deionized water and elution with 8 mL of methanol. The methanolic eluates were N_2 evaporated, and the residue from each was dissolved in 1.6 mL of aqueous 10% methanol including 0.02% phosphoric acid and transferred to 2 mL amber HPLC vials in preparation for HPLC separation of the caffeoylquinic acid derivatives.

HPLC isolation and purification of caffeoylquinic acid derivatives **1** and **2** was performed on a 250 mm × 4.6 mm i.d., 5 μm, Luna C18(2) analytical column (Phenomenex) using an HP 1100 series instrument (Agilent Technologies) as described by Ma et al.[25] A binary mobile phase gradient of methanol in 0.01% aqueous phosphoric acid was used as follows: 0−5 min, isocratic 10% methanol, 1.0 mL/min; 5−15 min, linear increase from 10 to 25% methanol, 1.0 mL/min; 15−25 min, linear increase from 25 to 50% methanol, 1.0 mL/min; 25−28 min, linear increase from 50 to 80% methanol, 1.0 mL/min; 28−30 min, 80% methanol, linear increase from 1.0 to 1.2 mL/min; 30−33 min, linear decrease from 80 to 10% methanol, 1.2 mL/min; 33−36 min, 10%

Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1 and 2 Isolated from *Solanum incanum* and Wild *Solanum melongena* Fruit Tissues

	1		2	
position	δ_H (int, mult, *J* in Hz)	δ_C	δ_H (int, mult, *J* in Hz)	δ_C
caffeoyl group				
1		126.4 s		126.4 s
2	6.99 (1H, d, 1.9)	113.8 d	6.98 (1H, d, 1.9)	113.9 d
3		145.4 s		145.4 s
4		148.2 s		148.3 s
5	6.72 (1H, d, 8.1)	115.1 d	6.71 (1H, d, 8.2)	115.1 d
6	6.90 (1H, dd, 8.1, 1.9)	121.6 d	6.90 (1H, dd, 8.3, 1.9)	121.7 d
7	7.50 (1H, d, 15.9)	145.8 d	7.47 (1H, d, 15.9)	146.1 d
8	6.19 (1H, d, 15.9)	113.7 d	6.15 (1H, d, 15.9)	113.4 d
9		166.9 s		166.4 s
quinic acid group				
1′		76.1 s		75.7 s
2′ax	2.25 (1H, dd, 14.0, 4.0)	36.3 t	2.23 (1H, dd, 14.3, 3.4)	37.5 t
2′eq	2.11 (1H, m)	36.3 t	2.11 (1H, m)	37.5 t
3′	5.32 (1H, ddd, 6.6, 6.3, 3.6)	72.3 d	4.29 (1H, ddd, 6.2, 6.0, 3.3)	67.4 d
4′	3.88 (1H, dd, 7.8, 3.3)	69.0 d	5.02 (1H, dd, 8.5, 3.0)	74.8 d
5′	5.26 (1H, ddd, 7.0, 6.9, 5.2)	70.5 d	5.46 (1H, ddd, 8.5, 8.4, 5.6)	67.4 d
6′ax	2.07 (1H, dd, 14.4, 6.7)	34.4 t	1.99 (1H, dd, 14.3, 5.6)	36.9 t
6′eq	2.12 (1H, m)	34.4 t	2.11 (1H, m)	36.9 t
7′		175.9 s		175.0 s
malonic acid group				
1″		167.1 s		167.9 s
2″	3.29 (2H, s)	48.2 t	3.29 (2H, s)	48.7 t
3″		170.6 s		170.0 s

methanol, linear decrease from 1.2 to 1.0 mL/min. The 5-*O*(*E*)- and 4-*O*(*E*)-caffeoylquinic acid isomers eluted at 21.8 and 23.2 min, respectively, whereas unknown caffeoylquinic acid derivatives **1** and **2** eluted at 25.2 and 26.7 min, respectively. Injection of 60−80 μL aliquots achieved optimal column loading and peak separation. Pooled fractions from the first round of collection of compounds **1** and **2** were N_2 evaporated to 4 mL. They were then loaded on 200 mg Strata-X tubes, which were washed with 3 mL of water and eluted with 6 mL of methanol. Solvent was N_2 evaporated, and the samples were prepared as described above for a second round of HPLC separation and purification. This yielded 3.27 and 1.72 mg of compounds **1** and **2**, respectively, at purities of 94 and 96%.

Analytical Procedures and Instrumentation. Optical rotations, UV and IR spectra, ^{1}H, 2D, and ^{13}C NMR experiments, and high-resolution electrospray ionization mass spectroscopy (HR-ESI-MS) were all obtained or conducted using the same instruments and procedures as those previously described in detail by Ma et al.[25]

Hydroxycinnamic acid conjugate (1): 1β,4β-dihydroxy-3β-carboxyacetoxy-5α-[(*E*)-3-(3,4-dihydroxyphenyl)acryloyloxy]-cyclohexanecarboxylic acid (IUPAC numbering): white amorphous powder; IR ν_{max} cm^{-1}, 3356, 2922, 2357, 1652, 1532, 1395, 1281, 1164; UV (CH_3OH) λ_{max} nm (log ε), 328 (3.72), 300sh (3.61), 218 (3.69); $[\alpha]_D^{20} = -48.3°$ (*c*, 0.00029, MeOH); ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 75 MHz) data, see Table 1; HR-ESI-MS (negative mode), *m*/*z* 439.0876 ($[M - H]^-$ calcd for $C_{19}H_{19}O_{12}$, 439.0877).

Hydroxycinnamic acid conjugate (2): 1β,3β-dihydroxy-5α-carboxyacetoxy-4β-[(*E*)-3-(3,4-dihydroxyphenyl)acryloyloxy-cyclohexanecarboxylic acid: white amorphous powder; IR ν_{max} cm^{-1}, 3381, 2922, 2364, 1701, 1533, 1398, 1283, 1166; UV (CH_3OH) λ_{max} nm (log ε), 329 (3.60), 300sh (3.50), 217 (3.59); $[\alpha]_D^{20} = -42.9°$ (*c*, 0.00014, MeOH); ^{1}H NMR (CD_3OD, 300 MHz) and ^{13}C NMR (CD_3OD, 75 MHz) data, see Table 1; HR-ESI-MS (negative mode), *m*/*z* 439.0877 ($[M - H]^-$ calcd for $C_{19}H_{19}O_{12}$, 439.0877).

DPPH Free Radical (DPPH•) Scavenging Activity. DPPH• scavenging activity was assessed using the method described by Smith et al.[20] with minor modifications. To a 50 μL aliquot of the sample was added 150 μL of DPPH• (400 μM). The absorbance at 515 nm was recorded after initial mixing and subsequently after 30 min of incubation at 37 °C using a Molecular Devices $Versa_{max}$ microplate reader (Sunnyvale, CA). The percentage of DPPH• scavenging for different concentrations of the compounds was calculated using eq 1:

$$\text{scavenging percentage} = \left[\frac{(\text{absorbance}_{\text{control}}) - \text{absorbance}_{\text{sample}})}{(\text{absorbance}_{\text{control}})}\right] \times 100$$

A plot of the percentage of DPPH• scavenging versus concentration was made for the reference standard, Trolox. On the basis of this plot the Trolox equivalent antioxidant capacity (TEAC, mM Trolox/mM compound) values for different samples were calculated.

ABTS Free Radical ($ABTS^{•+}$) Scavenging Activity. Determination of $ABTS^{•+}$ scavenging was carried out using the method of Re et al.[21] $ABTS^{•+}$ was generated by reacting ABTS in aqueous solution (7 mM) with aqueous $K_2S_2O_8$ (2.45 mM) in the dark at ambient temperature for 12−16 h and then adjusting the $A_{734\ nm}$ to 0.700 (±0.020) with ethanol. To a 2 μL aliquot of the sample was added 198 μL of $ABTS^{•+}$. Absorbance at 734 nm was recorded after initial mixing and subsequently at 5 min intervals (for 40 min in total) with a Molecular

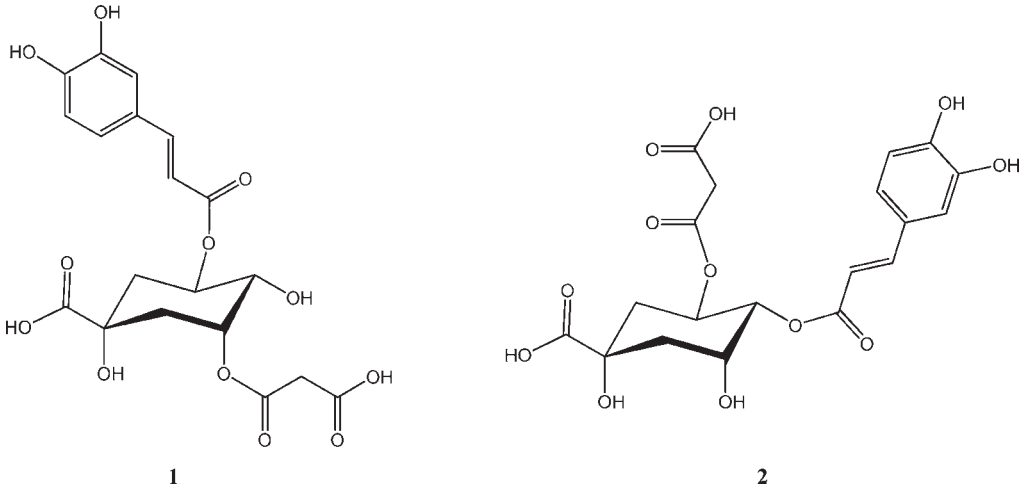

Figure 1. Structures of new malonylcaffeoylquinic acid isomers **1** and **2** isolated from fruit tissues of *Solanum incanum* and wild *Solanum melongena*.

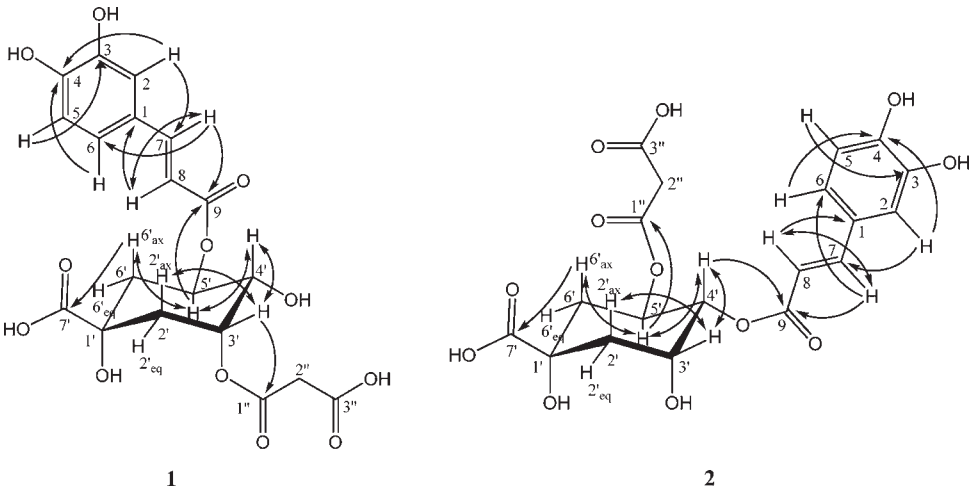

Figure 2. Selected HMBC (→) and H-HCOSY (↔) NMR correlations used in structural elucidation of malonylcaffeoylquinic acid isomers **1** and **2** isolated from fruit tissues of *Solanum incanum* and wild *Solanum melongena*.

Devices $Versa_{max}$ microplate reader. The results were expressed as TEAC (mM Trolox/mM compound) values at different time intervals.

Iron Chelation Activity. Iron chelation activity was assessed using the method of Carter[22] with minor modification. To 20 μL of the sample were added 10 μL of iron(II) chloride tetrahydrate (2 mM) and 90 μL of methanol. The reaction mixture was incubated for 5 min; thereafter, 40 μL of ferrozine (5 mM) was added. After 10 min, the absorbance was measured at 562 nm, using a Molecular Devices $Versa_{max}$ microplate reader. The percentage of chelation was calculated using eq 2:

$$\text{percentage of chelation} = \left(\frac{\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}}\right) \times 100$$

A plot of the percentage of chelation versus concentration was made for the chosen reference standard, Na_2EDTA. The results were expressed as Na_2EDTA equivalent (mM Na_2EDTA/mM compound) values.

Statistical Analysis. Data are presented as mean values ± 95% confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $P < 0.05$. JMP 8 software (SAS Institute, Inc., Cary, NC) was used for statistical analyses.

RESULTS AND DISCUSSION

A molecular formula of $C_{19}H_{20}O_{12}$ for compound **1** (Figure 1) was determined from the HR-ESI-MS molecular ion $[M - H]^-$. The UV spectrum of **1** showed absorption maxima at 328, 300sh, and 218 nm.

The ^{1}H NMR signals at δ 6.72 (1H, d, J = 8.1 Hz, H-5), δ 6.99 (1H, d, J = 1.9 Hz, H-2), and δ 6.90 (1H, dd, J = 8.1, 1.9 Hz, H-6) suggested the presence of a set of aromatic ABX system protons. The ^{1}H NMR doublets at δ 7.50 (1H, d, J = 15.9 Hz, H-7) and δ 6.19 (1H, d, J = 15.9 Hz, H-8) and the H−H COSY cross-peak H-7/H-8 indicated two *trans* oriented olefinic protons (Figure 2). From the HMBC correlations H-7/C-6(δ 121.6), C-9 (δ 166.9), H-8/C-1 (δ 126.4), H-2, H-6/C-4 (δ 148.2), and H-5/C-3(δ 145.4) (Figure 2), a caffeoyl group was deduced in the structure. The ^{13}C NMR signals at δ 115.1 (C-5), δ 113.8 (C-2), δ 121.6 (C-6), δ 145.8 (C-7), and δ 113.7 (C-8) were assigned from HSQC data.

A quinic acid moiety was indicated by ^{1}H NMR resonances of three oxymethine protons at δ 5.32 (1H, ddd, J = 6.6, 6.3, 3.6 Hz, H-3′), δ 3.88 (1H, dd, J = 7.8, 3.3 Hz, H-4′), and δ 5.26 (1H, ddd,

J = 7.0, 6.9, 5.2 Hz, H-5′), together with two pairs of sp^3 methylene protons at δ 2.11 (1H, m, H-2′$_{eq}$)/2.25(1H, dd, J = 14.0, 4.0 Hz, H-2′$_{ax}$) and δ 2.12 (1H, m, H-6′$_{ax}$)/2.07 (1H, dd, J = 14.4, 6.7 Hz, H-6′$_{eq}$) as shown in Table 1. By inspection of the ^{13}C NMR and DEPT spectra, these resonances were in agreement with three oxymethine resonances at δ 72.3 (C-3′), δ 69.0 (C-4′), and δ 70.5 (C-5′) and two sp^3 methylenes at δ 36.3 (C-2′) and δ 34.4 (C-6′). Their assignments were determined from HSQC data. Moreover, there was an oxygenated quaternary carbon at δ 76.1 (C-1′) and a carboxyl resonance at δ 175.9 (C-7′) in the ^{13}C NMR spectrum, which are also characteristic of quinic acid. The assignments for the quinic acid nucleus were confirmed by analysis of the H−H COSY cross-peaks H-2′$_{ax}$/H-3′, H-3′/H-4′, H-4′/H-5′ and H-5′/H-6′$_{ax}$ and HMBC correlation H-6′$_{ax}$/C-7′ (Figure 2). The deshielded resonances of two oxymethine protons in the quinic acid nucleus at δ 5.32 (H-3′) and δ 5.26 (H-5′) implied acylation of the hydroxyl groups at these positions as reported for other natural quinic acid derivatives.[23,24] The HMBC correlation between H-5′/C-9 further confirmed that OH-5′ on quinic acid is esterified to caffeic acid.

All of the remaining signals, including δ 167.1 (C-1″), δ 48.2 (C-2″), and δ 170.6 (C-3″) in the ^{13}C NMR spectrum and t δ 3.29 (2H, s, H-2″) in the ^{1}H NMR spectrum, were attributed to a propanedioic acid (malonyl) group. The HMBC correlation between H-3′ and C-1″ demonstrated that the propanedioic acid group is attached to OH-3′ of the quinic acid moiety.

Additionally, ToF MS/MS yielded the fragments m/z 395.0970 $[C_{18}H_{19}O_{10}, (M - H) - COO]^-$, m/z 353.0887 $[C_{16}H_{17}O_9, (M - H) - C_3H_2O_3$ (propanedioic acid)$]^-$, and m/z 335.0775 $[C_{16}H_{15}O_8, (M - H) - C_3H_2O_3$ (propanedioic acid) $- H_2O]^-$, which verified the structure assigned by NMR analyses. Thus, the structure of **1** was determined to be 1β,4β-dihydroxy-3β-carboxyacetoxy-5α-[(*E*)-3-(3,4-dihydroxyphenyl)acryloyloxy]cyclohexanecarboxylic acid (IUPAC numbering).

For compound **2** (Figure 1), the HR-ESI-MS molecular ion $[M - H]^-$ gave the molecular formula $C_{19}H_{20}O_{12}$, which is isomeric to **1**. The UV absorbance spectrum of **2** showed maxima at 329, 300sh, and 217 nm, very similar to those exhibited by **1**. On the basis of data from ^{1}H and ^{13}C NMR, H-HCOSY, HSQC, and HMBC experiments, the structure of **2** was determined to also comprise a caffeoyl group, a quinic acid, and a propanedioic acid (malonyl) moiety. The ^{1}H and ^{13}C NMR signals of **2** (Table 1) were nearly superimposable with those of **1**, except for chemical shifts of the H-3′ and H-4′ signals from quinic acid. The signals δ 4.29 (1H, ddd, J = 6.2, 6.0, 3.3 Hz, H-3′) and δ 5.02 (1H, dd, J = 8.5, 3.0 Hz, H-4′) in **2**, compared with δ 5.32 (1H, ddd, J = 6.6, 6.3, 3.6 Hz, H-3′) and δ 3.88 (1H, dd, J = 7.8, 3.3 Hz, H-4′) in **1**, indicated acylation at C-4′ in **2** rather than at C-3′ as in **1** (Table 1). The HMBC correlation H-4′/C-9 showed that the OH-4′ on quinic acid was esterified by caffeoyl group, and the HMBC correlation H-5′/C-1″ confirmed that the propanedioic acid group is linked to OH-5′ in **2**. The fragmental ions from the HR-ToF-ESI-MS spectrum of **2** including m/z 395.0982 $[C_{18}H_{19}O_{10}, (M - H) - COO]^-$, m/z 353.0873 $[C_{16}H_{17}O_9, (M - H) - C_3H_2O_3$ (propanedioic acid)$]^-$, and m/z 335.0767 $[C_{16}H_{15}O_8, (M - H) - C_3H_2O_3$ (propanedioic acid) $- H_2O]^-$, were closely similar to those of **1**. Thus, the structure of **2** was determined to be 1β,3β-dihydroxy-5α-carboxyacetoxy-4β-[(*E*)-3-(3,4-dihydroxyphenyl)acryloyloxy]cyclohexanecarboxylic acid.

Both compounds **1** and **2** demonstrated DPPH$^\bullet$ scavenging activity (Figure 3). The activity of **2** (1.65 ± 0.10 mM Trolox/mM compound) was significantly higher than that of **1** (1.34 ± 0.02 mM Trolox/mM compound). By comparison, the flavonol standard quercetin dihydrate showed about 3.5−4.4-fold higher scavenging activity (5.85 ± 0.12 mM Trolox/mM compound) than the malonylcaffeoylquinic acid isomers (Figure 3). Free radicals promote the progression of oxidative stress, and thus scavenging of these reactive species is an important mechanism of antioxidant action. Therefore, **1** and **2** may have the ability to reduce free radical mediated oxidative damage in vivo.

Compounds **1** and **2** also showed ABTS$^{\bullet+}$ scavenging activity for 35−40 min (Table 2). The scavenging activity of **1** increased

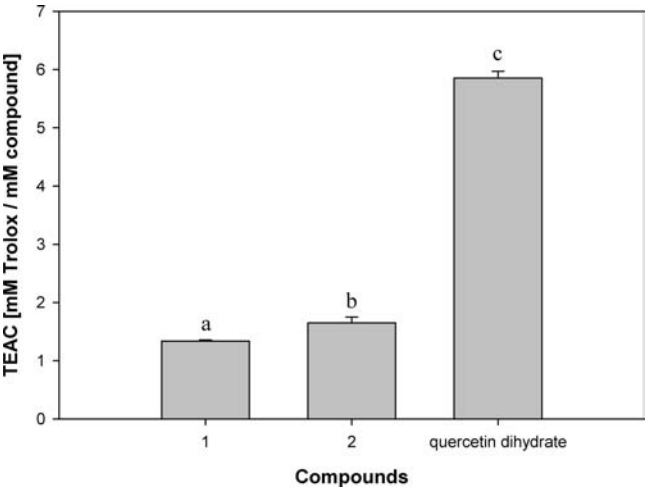

Figure 3. DPPH$^\bullet$ scavenging activity of malonylcaffeoylquinic acid isomers **1** and **2** isolated from fruit tissues of *Solanum incanum* and wild *Solanum melongena*. Values are expressed as the mean ± 95% confidence interval (n = 6). Bars with different letters (a−c) are significantly (P < 0.05) different. Analysis of variance was performed by ANOVA procedures, with significant differences between means determined by Tukey's pairwise comparisons.

Table 2. ABTS$^{\bullet+}$ Scavenging Activity of Compounds 1 and 2 from Fruit Tissues of *Solanum incanum* and Wild *Solanum melongena*

	ABTS$^{\bullet+}$ scavenging (TEAC)[a]		
time (min)	quercetin	**1**	**2**
0	1.32 ± 0.11 A,a	0.64 ± 0.05 A,b	0.66 ± 0.07 A,b
5	1.77 ± 0.10 B,a	0.66 ± 0.04 A,B,b	0.68 ± 0.07 A,B,b
10	1.88 ± 0.10 B,C,a	0.68 ± 0.04 A,B,C,b	0.74 ± 0.06 A,B,C,b
15	1.95 ± 0.10 B,C,a	0.71 ± 0.04 A,B,C,D,b	0.80 ± 0.06 B,C,D,b
20	1.98 ± 0.09 C,a	0.74 ± 0.04 B,C,D,E,b	0.86 ± 0.06 C,D,E,c
25	2.01 ± 0.08 C,a	0.77 ± 0.04 C,D,E,b	0.92 ± 0.06 D,E,F,c
30	2.04 ± 0.07 C,a	0.80 ± 0.04 D,E,b	0.99 ± 0.05 E,F,G,c
35	2.06 ± 0.07 C,a	0.82 ± 0.04 E,b	1.04 ± 0.05 F,G,c
40	2.08 ± 0.06 C,a	0.83 ± 0.04 E,b	1.09 ± 0.05 G,c

[a] Values are expressed as the mean ±95% confidence intervals (n = 6). Values with different lower case letters (a−c) within each row and upper case letters (A−G) within each column are significantly different (P < 0.05). Analysis of variance was performed by ANOVA procedures, with significant differences between means determined by Tukey's pairwise comparisons. Trolox equivalent antioxidant capacity (TEAC) is expressed as mM Trolox/mM compound.

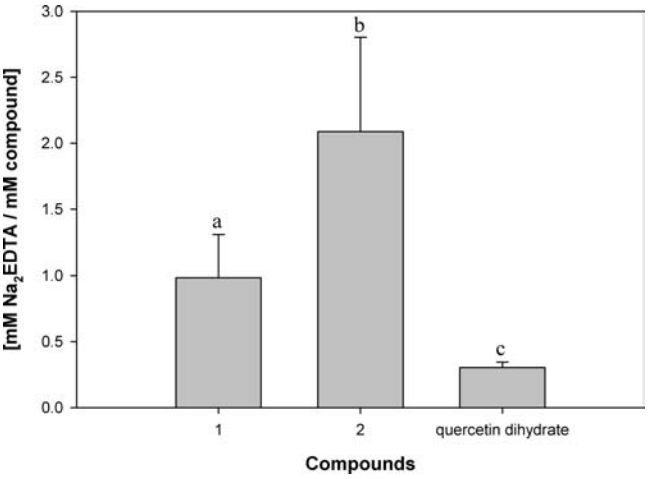

Figure 4. Iron chelation activity of malonylcaffeoylquinic acid isomers **1** and **2** isolated from fruit tissues of *Solanum incanum* and wild *Solanum melongena*. Values are expressed as the mean ± 95% confidence interval (n = 6). Bars with different letters (a−c) are significantly (P < 0.05) different. Analysis of variance was performed by ANOVA procedures, with significant differences between means determined by Tukey's pairwise comparisons.

up to 35 min, after which there was no significant additional increase. By contrast, the activity of **2** increased throughout the assay (40 min). The scavenging properties of the two compounds were not significantly different (P > 0.05) for up to 20 min. However, from 20 min onward the activity of **2** was significantly higher than that of **1**. The scavenging activity of quercetin dihydrate, the positive control, ranged from about 2- to 3-fold higher than that of both malonylcaffeoylquinic acid isomers throughout the assay.

Both the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays employ stable radical species to measure the electron-donating abilities of compounds and are well suited for assessment of the relative antioxidant capacity of small phenolics.[26] Comparison of results from the two assays, DPPH$^{\bullet}$ involving a fixed time measurement and ABTS$^{\bullet+}$ including determinations at 5 min intervals up to 40 min, provided a more complete picture of the radical scavenging properties of the malonated caffeoylquinic acid isomers. Although in terms of TEAC the two assays yielded substantially different values, both showed that **2** is a significantly more potent scavenger than **1**, but the positive control quercetin dihydrate was considerably more active than either isomer **1** or **2**.

As shown in Figure 4, the iron chelation activities of **1** (0.98 ± 0.32 mM Na_2EDTA/mM compound) and **2** (2.09 ± 0.72 mM Na_2EDTA/mM compound) were more than 3- and 6-fold higher, respectively, than that of the quercetin dihydrate control (0.30 ± 0.04 mM Na_2EDTA/mM compound). Iron chelation activity was also significantly higher for **2** compared with **1**. Transition metal ions, in particular iron(II), can cause the formation of ROS in vivo, thus promoting oxidative stress. As shown previously for phenolic acids including a 3,4-dihydroxy (catechol) moiety,[27] compounds **1** and **2** efficiently formed chelates with iron ions, which in vivo could prevent generation of ROS via what is termed the secondary antioxidant effect.

The only prior report of malonylcaffeoylquinic acid compounds within the Solanaceae was our isolation from *Solanum viarum* fruit of 3-*O*- and 4-*O*-malonyl isomers of 5-*O*(*E*)-caffeoylquinic acid, which also included a 6-*O*-sinapoylglucose group 1-β-D-linked with the 4-hydroxyl on the phenyl ring of the caffeoyl moiety.[25] An isomer of compounds **1** and **2**, 4-*O*-malonyl-5-*O*(*E*)-caffeoylquinic acid (current nomenclature), was previously isolated from leaves of *Albizia julibrissin* (Fabaceae).[28] Interestingly, the compound was proposed to serve as part of a blue light photoreceptor complex. Plant species in the Asteraceae include the greatest diversity of chlorogenic acids and derivatives,[12] so it is not surprising that a variety of malonylcaffeoylquinic acids have been reported in medicinal plants of this family.[29,30] The phenolic profiles of both *Erigeron breviscapus* [29] and *Helichrysum devium* [30] were determined entirely by LC/ESI-MS^n analyses, leaving some question about the linkages in the various malonated mono-, di-, and tri-*O*-caffeoylquinic acid isomers. In particular, two malonylmonocaffeoylquinic acids were found in *E. breviscapus*, but their exact structures were not determined, whereas for all malonyldicaffeoylquinic acids from *H. devium* it was inferred (without substantiation) that malonation occurs on the 4-hydroxyl of a caffeoyl moiety.

Several other recent reports of chlorogenic acid derivatives in medicinal plants from the Asteraceae, for example, refs 31 and 32, list an array of hydroxycinnamoylquinic acids that include one or more methoxyoxalic acid moieties esterified to hydroxyls on quinic acid. HPLC analysis of *Arnica montana* extracts after alkaline hydrolysis confirmed the presence of methoxyoxalic acid,[31] but in studies where identification of hydroxycinnamoylquinic acids derivatives relied solely on LC/ESI-MS^n data, for example, refs 29, 30, and 32, methoxyoxalyl and malonyl moieties cannot be differentiated.[30] Consequently, the distribution of methoxyoxalated versus malonated chlorogenic acids among species of the Asteraceae and possibly other plant families is uncertain. The importance of complete structural elucidation lies in determining precisely which compounds are responsible for a specific biological activity. A good example is the recent demonstration by Di Paola et al.[15] that 3,5-dicaffeoyl-4-malonylquinic acid isolated from the medicinal plant *Centella asiatica* may be beneficial for treatment of inflammatory bowel disease. Currently it is not known if any isomer of malonylmonocaffeoylquinic acid exhibits similar or other biological activity. Future research is planned for in vitro testing of the potential biological activity of compounds **1** and **2**, as well as the 3-*O*-malonyl- and 4-*O*-malonyl-5-*O*(*E*)-(6-sinapoylglucosyl)caffeoylquinic acid isomers isolated from *S. viarum*.[25] In addition, a BAHD acyltransferase that uses malonyl-CoA as a substrate is likely to catalyze the malonation of caffeoylquinic acids, and the availability of three eggplant germplasm accessions known to accumulate substantial levels of malonylcaffeoylquinic acids could facilitate cloning of the gene encoding this enzyme.

AUTHOR INFORMATION

Corresponding Author

*Phone: (301) 504-6984. Fax: (301) 504-5107. E-mail: bruce.whitaker@ars.usda.gov.

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