Activity-Guided Isolation of Antioxidative Constituents of *Cotinus coggygria*

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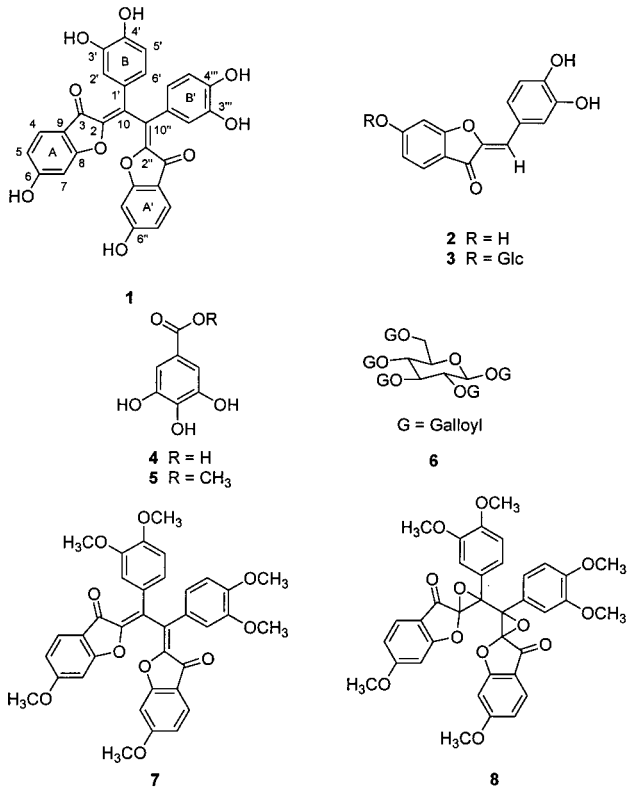
Six constituents (**1**−**6**) were isolated from EtOAc-soluble partitions of two separate collections of the whole plants of *Cotinus coggygria*, namely, disulfuretin {2,2′-[1,2-bis(3,4-dihydroxyphenyl)-1,2-ethanedylidene]-bis[6-hydroxy-3(2*H*)-benzofuranone] (**1**)}, sulfuretin (**2**), sulfurein (**3**), gallic acid (**4**), methyl gallate (**5**), and pentagalloyl glucose (**6**). The structure of the novel biaurone **1** was determined by spectral and chemical methods. Compounds **1**−**6** were found to be potent antioxidants in a 1,1-diphenyl-2-picrylhydrazyl free-radical scavenging assay.

It has been suggested that nearly 90% of all cancers are caused by potentially controllable factors.[1] Cancer chemoprevention involves the prevention, delay, or reversal of cancer by dietary or pharmaceutical agents capable of modulating the process of carcinogenesis.[2,3] Free radicals have been implicated in a number of diseases, including brain disorders, atherosclerosis, and colon cancer.[4] Endogenous and exogenous free radicals can cause DNA damage and have been directly correlated with increased cancer risk.[5] Although the estimated number of oxidative attacks on DNA in humans is daunting (10 000 per cell per day),[6] dietary antioxidants may offer some protection to those at greatest risk from oxidative damages by reducing cancer risk.[7] Antioxidants scavenge oxygen radicals, including singlet oxygen, peroxy radicals, superoxide anion, and hydroxyl radicals.[8,9]

Cotinus coggygria Scop. (Anacardiaceae) is a shrubby tree commonly known as the "smoke tree". It is an ornamental addition to many gardens throughout the United States.[10] Formerly, this species was classified in the genus *Rhus*, but, in 1965, Barkley split *Rhus sensu lato* into nine segregate genera of which one is *Cotinus* Mill. (*C. coggygria*).[11,12]

The current investigation was undertaken as part of a program to discover novel cancer chemopreventive agents from plants.[13] Two collections of the whole plants of *C. coggygria* were made in this study, with both showing in vitro antioxidative activity. The ethyl acetate partition (IC_{50} = 10.7 μg/mL) of the second *C. coggygria* collection was found to exhibit significant scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals.[14] Bioassay-guided fractionation of the active extract using the DPPH in vitro antioxidant assay as a monitor led to the isolation of three potent aurones (**1**−**3**), including 2,2′-[1,2-bis(3,4-dihydroxyphenyl)-1,2-ethanedylidene]bis-[6-hydroxy-3(2*H*)-benzofuranone] (disulfuretin, **1**), a novel compound. Activity-guided fractionation of the initial collection of *C. coggygria* (EtOAc-soluble extract, IC_{50} = 6.0 μg/mL in the DPPH antioxidant assay) led to the isolation of gallic acid (**4**), methyl gallate (**5**), and pentagalloyl glucose (**6**) as active constituents. Compounds **1**−**6** were compared with ascorbic acid and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) in the DPPH antioxidant assay.[15,16]

The ethyl acetate partition of *C. coggygria* (second collection, obtained in 1998) was chromatographed by passage over a Si gel column and was then subjected to further purification using a Sephadex LH-20 column, with the DPPH assay employed as a monitor. Three active aurones (**1**−**3**) were isolated, of which **1** is a new compound. Sulfuretin (**2**) has been isolated previously from *C. coggygria*,[12] but sulfurein (**3**)[17] has not been reported previously from this species.



Compound **1**, which was isolated as a bright yellow-green powder, displayed a characteristic aurone UV spectrum with absorption maxima at λ_{max} 255, 335, and 408 nm.[18] The molecular formula of **1** was deduced as $C_{30}H_{18}O_{10}$ by elemental analysis. The APT, HMBC, and HMQC NMR spectra were consistent with a biaurone skeleton with 12 methines and 18 quaternary carbons. Peaks at *m*/*z* 539, 270 (base peak), and 137 in the positive-ion electrospray tandem mass spectrum (ESIMS/MS) of the protonated molecule were consistent with **1** being a dimer of sulfuretin (**2**). The APT, COSY, HMQC, and HMBC NMR data

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Table 1. ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, and ^{1}H−^{13}C HMBC NMR Data for Compound **1**[a]

position	δ_H[a] (mult., *J* Hz)	^{13}C[b]	^{1}H−^{1}H COSY	^{1}H−^{13}C HMBC correlations
2		142.8		
3		181.0		
4	7.59 (d, 8.5)	126.4	H-5	C-3, C-5, C-6, C-8, C-9
5	6.48 (d, 8.5)	110.3	H-4	C-4, C-6, C-7, C-9
6		167.0		
7	6.72 (s)	98.8		C-5, C-6, C-8, C-9
8		172.8		
9		115.2		
10		147.7		
1′		126.1[c]		
2′	7.52 (s)	115.6		C-3′, C-5′, C-6′, C-10
3′		145.2		
4′		146.4		
5′	7.11 (d, 8.5)	117.7	H-6′	
6′	7.30 (dd, 8.5)	126.1	H-5′	C-2′, C-4′, C-10
2″		143.6		
3″		182.1		
4″	7.62 (d, 8.5)	126.7	H-5″	C-3″, C-5″, C-6″, C-8″, C-9″
5″	6.58 (d, 8.5)	110.3	H-4″	C-4″, C-6″, C-7″, C-9″
6″		168.8		
7″	6.76 (s)	99.2		C-5″, C-6″, C-8″, C-9″
8″		174.2		
9″		115.6		
10″		147.8		
1‴		126.1[c]		
2‴	7.56 (s)	119.8		C-3‴, C-5‴, C-6‴, C-10″
3‴		145.2		
4‴		146.5		
5‴	7.14 (d, 8.5)	118.1	H-6‴	
6‴	7.38 (dd, 8.5)	126.2	H-5‴	C-2‴, C-4‴, C-10″

[a] TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values in parentheses; measured at 300 MHz in $(CD_3)_2CO$. [b] Measured at 75 MHz in $(CD_3)_2CO$. [c] Overlapping signals.

showed an absence of connectivities on the A or B rings of both monomeric halves of **1**. The absence of H-10 proton signals in the ^{1}H NMR spectrum of **1** suggested that the intermolecular connection was at C-10 in both halves of the dimer. This was confirmed by the absence of any methine signal in the APT NMR spectrum and the presence of two additional quaternary carbons at δ 147.7 and 147.8 with exclusive HMBC connectivities to H-2′ (H-2‴) and H-6′ (H-6‴) in each half of the dimer. HMBC experiments allowed the complete assignments of the ^{1}H and ^{13}C NMR spectra of **1**, and a summary of all such correlations observed for **1** is shown in Table 1.

It was still necessary to locate more precisely the interauronyl connectivity between both halves of **1**. To determine if the connection involved an oxygen−carbon linkage, compound **1** was permethylated to produce compound **7**. The protonated molecule of *m*/*z* 625 in the positive ESIMS and the ^{1}H NMR spectrum of the permethylated derivative confirmed the presence of six methoxyl groups. Thus, the interauronyl linkage point could not have occurred through an oxygen−carbon linkage. The possibility that a biinterauronyl connection at C-2 (C-2″) and C-10 (C-10″) with an ethylene bridge was ruled out after compound **7** was diepoxidized to produce **8**, indicating the presence of two double bonds rather than a single double bond in a nonaromatic system. LC−MS confirmed the presence of a diepoxidized product with a protonated molecule at *m*/*z* 657. These data require that the interdisulfuretin linkage point in **1** be through a sigma bond between C-10 and C-10″. The stereochemistry of compound **1** suggested *Z* geometry for both halves of the molecule on the basis of the ^{13}C NMR chemical shifts for C-2 (C-2″), C-3 (C-3″), and C-9 (C-9″).[19] Storage (approximately 1 month) of **1** at an ambient temperature resulted in its conversion to **2**. Thus, **1** was assigned the structure 2,2′-[1,2-bis(3,4-dihydroxyphenyl)-1,2-ethanedylidene]bis[6-hydroxy-3(2*H*)-benzofuranone] and accorded the trivial name disulfuretin.

Table 2. Activities of Compounds **1**−**6** from *C. coggygria* in a DPPH Free-Radical Scavenging Assay

compound	IC_{50} (μg/mL)
1	9.7
2	16.1
3	18.7
4	10.0
5	18.6
6	12.6
ascorbic acid[a]	22.0
2(3)-*tert*-butyl-4-hydroxyanisole (BHA)[a]	21.0

[a] Control compound.

Compounds **4**−**6** were isolated using repeated Si gel column chromatography from an initial collection of *C. coggygria* made in 1972 and were identified as gallic acid,[20,21] methyl gallate,[22,23] and pentagalloyl glucose,[24] respectively.

Compounds **1**−**6** from the two *C. cogggyria* collections showed significant antioxidative activity in the DPPH assay, with **1** being the most potent of the six isolates obtained (Table 2).

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. UV spectra were obtained with a Beckman DU-7 spectrometer. IR spectra were obtained with a Midac Collegian FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with TMS as an internal standard using a Varian XL-300 instrument operating at 300 and 75.4 MHz, respectively. ^{1}H−^{1}H COSY NMR experiments were performed on this same instrument, using standard pulse sequences. APT, ^{1}H−^{13}C HMBC, and HMQC NMR experiments were conducted on a Bruker DPX-300 MHz spectrometer. Elemental analysis was conducted on a Hewlett-Packard EA 1108 CHN analyzer. High-performance liquid chromatography (HPLC)−ESIMS/MS was carried out using a Micromass Quattro II mass spectrometer (Manchester, U.K.) with argon as the collision gas and a collision energy of 35 eV. HPLC separations were carried out using an Applied Biosystems (Foster City, CA) Model 140A HPLC pump equipped with a Vydac (Hesperia, CA) C_{18} column (2 × 250 mm).

Plant Material. Two collections were made of the entire plant of *C. coggygria*. The first was collected by Mr. J. E. Catellina and Mr. S. Totura at Shawnee National Forest, Makanda, IL, in August, 1972 (voucher herbarium specimen no. 2180364; deposited at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL). The second collection was made by Mr. K. Allen at the Morton Arboretum, Lisle, IL, in September, 1998 (voucher specimen 143781, deposited at the Morton Arboretum).

Assay for DPPH Free-Radical Scavenging Potential. This antioxidant monitoring assay is based on the scavenging activity of stable DPPH free radicals.[25] Reaction mixtures containing test samples (dissolved in DMSO) and 300 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37 °C for 30 min. Absorbances were then measured at 515 nm, and percent inhibition by sample treatment was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH free radicals.

Extraction and Isolation. The dried plant material (850 g, 1972 collection; 2.2 kg, 1998 collection) was ground and extracted with MeOH−H_2O (9:1; 4 × 9 L) by maceration. The resultant extracts were combined and concentrated to dryness in vacuo at 30 °C. Each dried methanolic extract was partitioned with petroleum ether to afford a dried petroleum

ether-soluble residue (2.2 g first collection; 6.1 g second collection). Each methanolic extract was then suspended in H_2O and partitioned with EtOAc to give, upon drying, an EtOAc-soluble residue (2.9 g first collection; 5.8 g second collection) and an aqueous partition (3.1 g first collection; 6.5 g second collection).

Fractionation of the ethyl acetate partition (IC_{50} = 6.0 μg/mL in the DPPH assay) of the initial (1972) collection of plant material was carried out using an open Si gel column with a $CHCl_3$−MeOH−C_6H_6 gradient system (99:0:1→0:99:1). The eluates with TLC profiles of greatest similarity were combined into pooled fractions. Fractions F004 and F005, the only active fractions in the DPPH assay, were subjected to repeated fractionation using Sephadex LH-20 size-exclusion chromatography (MeOH) and yielded compounds **4** (0.0036% w/w), **5** (0.0014% w/w), and **6** (0.0033% w/w) in pure form.

Fractionation of the ethyl acetate partition (IC_{50} = 10.7 μg/mL in the DPPH assay) of the second collection of plant material (collected in 1998) was initiated by column chromatography over Si gel as stationary phase, using a gradient system with 90−20:5→40:4−39:1 $CHCl_3$−MeOH−Me_2CO−C_6H_6. The eluates containing similar TLC profiles were combined to provide 14 pooled fractions (fractions 4−17). Fraction 13 [eluted with $CHCl_3$−MeOH−Me_2CO−C_6H_6 (20:20:59:1)] was active in the DPPH antioxidant assay, and three aurones [**1** (0.00021% w/w), **2** (0.00099% w/w), and **3** (0.00022% w/w)] were obtained by repeated separation of this fraction over a column containing Sephadex LH-20 eluted with MeOH.

Disulfuretin (1): yellow-green powder; UV λ_{max} (EtOH) (log ϵ) 255 (3.9), 335 (4.2), 408 (4.4) nm; IR (film) ν_{max} 3450, 1890, 1610, 1600 cm^{-1}; ^1H NMR (300 MHz, CD_3OD), ^{13}C NMR (75.4 MHz, CD_3OD), and HMBC data, see Table 1; ESIMS−MS (positive-ion mode) (35 eV) *m/z* 539 $[M + H]^+$ (40), 271 (100), 137 (52). Anal. C, 66.86; H, 3.37. Calcd for $C_{30}H_{18}O_{10}$: C, 66.96; H, 3.37.

Sulfuretin (2): orange powder; ESIMS (positive-ion mode) *m/z* 271; UV, IR, ^1H NMR, and ^{13}C NMR data were consistent with literature values.[12,26,27]

Sulfurein (3): orange powder; ESIMS (positive-ion mode) *m/z* 433; UV, IR, ^1H NMR, and ^{13}C NMR data were consistent with literature values.[17]

Gallic acid (4): off-white powder; ESIMS (positive-ion mode) *m/z* 171; UV, IR, ^1H NMR, and ^{13}C NMR data were consistent with literature values.[20,21]

Methyl gallate (5): off-white powder; EIMS *m/z* 184; IR, ^1H NMR, and ^{13}C NMR data were consistent with literature values.[22,23,28]

Pentagalloyl glucose (6): off-white crystals; ESIMS (positive-ion mode) *m/z* 941; ^1H NMR and ^{13}C NMR data were consistent with literature values.[24]

Methylation of Compound 1. Disulfuretin (**1**, 5.1 mg) was dissolved in dry acetone (1 mL). Under a N_2 atmosphere, 11.0 mg of K_2CO_3 was added to the solution followed by 0.4 mL (9.1 mg) of iodomethane, and the mixture was stirred overnight. The reaction mixture was extracted with $CHCl_3$. After evaporation of the solvent under reduced pressure, a residue was obtained, which was finally purified on a Sephadex LH-20 column (MeOH) to afford the pure methylated compound **7** (2.5 mg): ^1H NMR (acetone-d_6, 300 MHz) δ 7.66 (1H, s, H-2‴), 7.65 (1H, d, H-4″), 7.63 (1H, s, H-2′), 7.62 (1H, s, H-4), 7.43 (1H, dd, H-6‴), 7.36 (1H, dd, H-6′), 7.05 (1H, d, H-5‴), 7.02 (1H, d, H-5′), 6.81 (1H, s, H-7″), 6.77 (1H, s, H-7), 6.24 (1H, d, H-5″), 6.14 (1H, d, H-5), 4.11 and 3.94 (18H, s, OCH_3); ESIMS (positive ion mode) *m/z* 625 $[M + H]^+$.

Epoxidation of Compound 7. Compound **7** (3.4 mg) was dissolved in 0.5 mL of CH_2Cl_2, and 1.1 mg of 50−60% 3-*m*-chloroperoxybenzoic acid was added under a N_2 atmosphere. The solution was stirred overnight, and the reaction mixture was dissolved in EtOAc (1.0 mL) and washed sequentially with H_2O, aqueous $NaHCO_3$, and aqueous NaCl (1.0 mL each). The organic phase was dried with anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Final purification of a residue was obtained using Sephadex LH-20 column chromatography (MeOH) to afford a pure epoxidized product, **8** (1.0 mg): ESIMS (positive-ion mode) *m/z* 657 $[M + H]^+$.

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