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Phytochemistry 63 (2003) 335–341
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Activity-guided isolation of the chemical constituents of *Muntingia calabura* using a quinone reductase induction assay

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Received 6 December 2002; received in revised form 24 January 2003

Abstract

Activity-guided fractionation of an EtOAc-soluble extract of the leaves of *Muntingia calabura* collected in Peru, using an in vitro quinone reductase induction assay with cultured Hepa 1c1c7 (mouse hepatoma) cells, resulted in the isolation of a flavanone with an unsubstituted B-ring, (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone (**5**), as well as 24 known compounds, which were mainly flavanones and flavones. The structure including absolute stereochemistry of compound **5** was determined by spectroscopic (HRMS, 1D and 2D NMR, and CD spectra) methods. Of the isolates obtained, in addition to **5**, (2*S*)-5-hydroxy-7-methoxyflavanone, 2′,4′-dihydroxychalcone, 4,2′,4′-trihydroxychalcone, 7-hydroxyisoflavone and 7,3′,4′-trimethoxyisoflavone were found to induce quinone reductase activity.

Keywords: *Muntingia calabura*; Elaeocarpaceae; (2*R*,3*R*)-*trans*-7-methoxy-3,5,8-trihydroxyflavanone; Quinone reductase induction assay

1. Introduction

Induction of Phase 2 drug-metabolizing enzymes such as quinone reductase (QR) is considered an effective strategy for achieving protection against the toxic and neoplastic effects of many carcinogens (Gerhäuser et al., 1997; Talalay, 2000). As part of our continuing search for novel, plant-derived cancer chemopreventive agents (Pezzuto, 1997; Kinghorn et al., 1998; Pezzuto et al., 1999), the leaves of *Muntingia calabura* L. (Elaeocarpaceae), collected in Peru, were chosen for detailed investigation since their EtOAc-soluble extract significantly induced QR with cultured Hepa 1c1c7 (mouse hepatoma) cells. Various parts of this tree have several documented medicinal uses in both Southeast Asia and tropical America (Kaneda et al., 1991; Nshimo et al., 1993). The roots have been employed as an emmenogogue in Vietnam and as an abortifacient in Malaysia. In the Philippines, the flowers of this species have been used to treat headaches, and as an antidyspeptic, antispasmodic, and diaphoretic. Infusions of the flowers of this plant are drunk as a tranquillizer and tonic in Colombia (Perez-Arbelaez, 1975; Kaneda et al., 1991).

In our previous work, several cytotoxic flavonoids and chalcones were isolated from the roots (Kaneda et al., 1991) and the leaves and stems (Nshimo et al., 1993) of this species collected in the Philippines and in Thailand, respectively. Other phytochemical investigations on this plant have resulted in the isolation of flavones and ellagic acid (Seetharaman, 1990). The volatile phenolic, sesquiterpene and furanoid constituents of the ripe fruits of *M. calabura* have been analyzed by GC–MS (Wong et al., 1996). This paper describes the isolation and structure elucidation of 24 known compounds, (**1**–**4**, **6**–**14**), and a new flavanone, (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone (**5**), obtained using the QR induction assay to monitor fractionation (Chang et al., 1997; Dinkova-Kostova and Talalay, 2000). The

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doi:10.1016/S0031-9422(03)00112-2

purified isolates from *M. calabura* were individually evaluated for their effects on QR induction.

2. Results and discussion

Fractionation of an EtOAc-soluble extract of the leaves of *M. calabura* with the QR induction assay led to the purification of a new flavanone, (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone (**5**), as well as 24 known compounds, (2*S*)-7-hydroxyflavanone (**1**) (Tanrisever et al., 1987; Hsieh et al., 1998a), (2*S*)-5,7-dihydroxyflavanone (pinocembrin, **2**) (Ichino et al., 1988; Hsieh et al., 1998), (2*R*,3*R*)-3,5,7-trihydroxyflavanone (pinobanksin, **3**) (Kuroyanagi et al., 1982), (2*S*)-5-hydroxy-7-methoxyflavanone (pinostrobin, **4**) (Ichino et al., 1988; González et al., 1989), 7-hydroxyflavone (Silva et al., 1994), 5,7-dihydroxyflavone (chrysin) (Yang et al., 1996), 3-methoxy-5,7,4′-trihydroxyflavone (isokaemferide) (Wang et al., 1989), 3,3′-dimethoxy-5,7,4′-trihydroxyflavone (Wang et al., 1989), 8-methoxy-3,5,7-trihydroxyflavone (**6**) (Karasartov et al., 1992), 3,8-dimethoxy-5,7,4′-trihydroxyflavone (Roitman and James, 1985), 3,5-dihydroxy-7,4′-dimethoxyflavone (ermanin) (Echeverri et al., 1991), 3,5-dihydroxy-7,8-dimethoxyflavone (gnaphaliin, **7**) (Haensel and Ohlendorf, 1969), 5-hydroxy-3,7,8-trimethoxyflavone (Proksch et al., 1982; Reinecke and Minter, 1994), 5,4′-dihydroxy-3,7,8-trimethoxyflavone (**8**) (Bernhard and Thiele, 1981), 5-hydroxy-3,7,8,4′-tetramethoxyflavone (**9**) (Pandey et al., 1984), 2′,4′-dihydroxychalcone (**10**) (Tanrisever et al., 1987; Barrero et al., 1997), 4,2′,4′-trihydroxychalcone (isoliquiritigenin, **11**) (Kitagawa et al., 1993), 7-hydroxyisoflavone (**12**) (Nishiyama et al., 1993), 7,3′,4′-trimethoxyisoflavone (cabreuvin, **13**) (Fonseca et al., 2000; Ohsaki et al., 1999), (2*S*)-5′-hydroxy-7,8,3′,4′-tetramethoxyflavan (Kaneda et al., 1991), 2′,4′-dihydroxydihydrochalcone (**14**) (Jain and Mehta, 1985), 3,4,5-trihydroxybenzoic acid, lupenone (Dantanarayana et al., 1982), and 2α,3β-dihydroxy-olean-12-en-28-oic acid (Ikuta et al., 1995). The structures of these known compounds were identified by physical and spectroscopic data measurement ($[\alpha]_D$, ^{1}H NMR, ^{13}C NMR, DEPT, 2D NMR, and MS) and by comparing the data obtained with those of published values. The ^{13}C NMR spectral data of compounds **6**–**9** and **14** were assigned for the first time (Table 1 and Experimental).

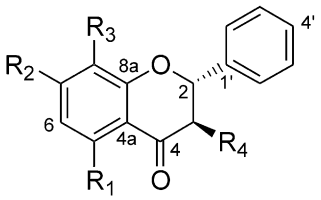
A literature survey revealed that most of the flavanones isolated from natural sources thus far have the 2*S* absolute configuration (Yoshikawa et al., 1998; Kuroyanagi et al., 1982), but 2*R* flavanones also have been reported (Hsieh et al., 1998b; Matsuda et al., 2002). However, divergent optical rotation values are occasionally reported for the same flavanone. For example, the specific rotation values of −3.4° (*c* 0.5, MeOH) (Ichino et al., 1988), −47.3° (*c* 5.48, MeOH) (Hsieh et al., 1998) and −52° (*c* 0.188, MeOH) (Fukui et al., 1988) were reported for 5,7-dihydroxyflavanone (**2**), and values of −1.6° (*c* 0.45, $CHCl_3$) (Ichino et al., 1988), −45° (*c* 0.50, $CHCl_3$) (Häberlein and Tschiersch, 1994) and +5° (*c* 0.05) (González et al., 1989) were reported for 5-hydroxy-7-methoxyflavanone (**4**). Values of −58.5° (*c* 0.91, MeOH) and −57.5° (*c* 0.80, $CHCl_3$) were obtained for compounds **2** and **4** in the present study, respectively. Furthermore, the enantiomeric mixture form of 5-hydroxy-7-methoxyflavanone was isolated from the roots of *Renealmia nicolaioides* in our recent work (Gu et al., 2002). In the CD spectra of compounds **1**–**4**, negative and positive Cotton effects, respectively, were observed in the π→π* and n→π transition regions (see Experimental). These observations indicated 2*S* absolute stereochemistry for flavanones **1**, **2** and **4** and 2*R*,3*R* stereochemistry for **3** (Gaffield, 1970).

Compound **5** was obtained as a yellowish amorphous powder, mp 226–228 °C, $[\alpha]_D^{23}$ +30.0° (*c* 0.25, $CHCl_3$ + MeOH, 1:1). A molecular formula of $C_{16}H_{14}O_6$ was assigned for **5** based on the protonated molecular ion peak at *m*/*z* 303.0866 $[M+H]^+$ (calc. for $C_{16}H_{15}O_6$, 303.0869) in its HRCIMS. The ^{1}H NMR spectrum of compound **5** displayed signals of two oxygenated methine doublets at δ_H 5.17 (1H, *d*, *J* = 11.1 Hz, H-2) and 4.62 (1H, *d*, *J* = 11.1 Hz, H-3), which were

Table 1
^{1}H NMR spectral data of **5** and ^{13}C and DEPT NMR spectral data of **5**–**9**[a]

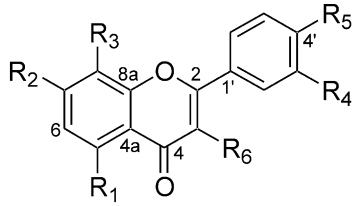
	5[b]		**6**[b]	**7**[c]	**8**[d]	**9**[d]
Position	δ_H	δ_C	δ_C	δ_C	δ_C	δ_C
2	5.17, *d* (11.1)	82.9 *d*	146.5 *s*	145.3 *s*	149.3 *s*	1493 *s*
3	4.62, *d* (11.1)	71.8 *d*	137.9 *s*	136.3 *s*	139.1 *s*	139.3 *s*
4		198.3 *s*	177.2 *s*	175.8 *s*	179.9 *s*	179.9 *s*
4a		101.0 *s*	104.0 *s*	103.3 *s*	105.9 *s*	105.9 *s*
5		155.2 *s*	156.1 *s*	156.4 *s*	156.9 *s*	156.6 *s*
6	6.26, *s*	92.8 *d*	99.2 *d*	95.2 *d*	96.3 *d*	96.3 *d*
7		157.2 *s*	157.5 *s*	158.7 *s*	158.7 *s*	159.6 *s*
8		147.7 *s*	128.4 *s*	129.1 *s*	129.8 *s*	129.8 *s*
8a		155.5 *s*	156.4 *s*	156.4 *s*	158.2 *s*	158.2 *s*
1′		137.3 *s*	132.0 *s*	130.9 *s*	122.8 *s*	123.8 *s*
2′	7.41-7.43, *m*	128.0 *d*	128.3 *d*	127.7 *d*	131.3 *d*	131.1 *d*
3′	7.54-7.56, *m*	128.5 *d*	129.5 *d*	128.7 *d*	116.5 *d*	115.1 *d*
4′	7.41-7.43, *m*	128.0 *d*	130.9 *d*	130.4 *d*	161.0 *s*	162.9 *s*
5′	7.54-7.56, *m*	128.5 *d*	129.5 *d*	128.7 *d*	116.5 *d*	115.1 *d*
6′	7.41-7.43, *m*	128.0 *d*	128.3 *d*	127.7 *d*	131.3 *d*	131.1 *d*
OH-5	11.59, *s*					
OMe	3.84 *s*	56.1 *q*	61.8 *q*	61.6 *q* (8)	61.6 *q* (8)	61.6 *q* (8)
				56.4 *q* (7)	60.2 *q* (3)	60.3 *q* (3)
					56.9 *q* (7)	56.9 *q* (7)
						55.9 *q* (4′)

[a] All data were assigned based on the observed 2D NMR spectral correlations.
[b] In DMSO-d_6.
[c] In $CDCl_3$.
[d] In acetone-d_6.



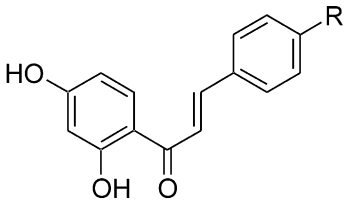
Flavanones

1 R_1=H R_2=OH R_3=H R_4=H
2 R_1=OH R_2=OH R_3=H R_4=H
3 R_1=OH R_2=OH R_3=H R_4=OH
4 R_1=OH R_2=OMe R_3=H R_4=H
5 R_1=OH R_2=OMe R_3=OH R_4=OH

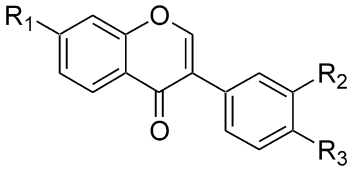


Flavones

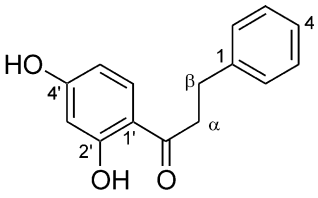
6 R_1=OH R_2=OH R_3=OMe R_4=H R_5=H R_6=OH
7 R_1=OH R_2=OMe R_3=OMe R_4=H R_5=H R_6=OH
8 R_1=OH R_2=OMe R_3=OMe R_4=H R_5=OH R_6=OMe
9 R_1=OH R_2=OMe R_3=OMe R_4=H R_5=OMe R_6=OMe



10 R=H
11 R=OH



12 R_1=OH R_2=R_3=H
13 R_1=R_2=R_3=OMe



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coupled to each other, an aromatic or olefinic singlet at δ_H 6.26 (1H, *s*, H-6), a methoxyl group at δ_H 3.84 (3H, *s*, OMe-7), and the multiplets of a mono-substituted aromatic ring at δ_H 7.54-7.56 (2H, *m*, H-3′ and H-5′) and 7.41–7.43 (3H, *m*, H-2′, H-4′ and H-6′). Also observed was a downfield exchangeable singlet at δ_H 11.59 (1H, *s*, OH-5), which is generally characteristic for the chelated OH-5 of flavones and flavanones. Consistent with the ^{1}H NMR spectral analysis, the ^{13}C NMR spectrum of compound **5** also displayed two oxygenated methines at δ_C 82.9 (C-2, *d*) and 71.8 (C-3, *d*), a methoxyl group at δ_C 56.1 (OMe, *q*), the signals of two aromatic rings (with one mono-substituted: δ_C 137.3, C-1′, *s*; δ_C 128.5, C-3′, C-5′, *d*; δ_C 128.0, C-2′, C-4′, C-6′, *d*), and a conjugated ketone at δ_C 198.3 (C-4, *s*). Accordingly, this suggested that compound **5** is a flavanone with an unsubstituted B ring (Kuroyanagi et al., 1982; Ichino et al., 1988). Thus, three hydroxyl groups in **5** could be inferred from the molecular formula of $C_{16}H_{14}O_6$, since only one methoxyl group was evident in both the ^{1}H and ^{13}C NMR spectra. In the HMBC spectrum of **5**, the downfield proton singlet of the chelated hydroxyl group at δ_H 11.59 (OH-5) correlated to the aromatic methine at δ_C 92.8 (C-6), and two quaternary carbons at δ_C 155.2 (C-5) and 101.0 (C-4a), indicating compound **5** is a 3,5,7,8-tetrasubstituted flavanone. The location of the methoxyl group at C-7 was assigned based on the observed NOESY correlation from δ_H 3.84 (OMe-7) to δ_H 6.26 (H-6). The coupling constant between H-2 and H-3 (11.1 Hz) suggested that the B ring and OH-3 were equatorially *trans*-oriented (Gaffield, 1970). The CD spectrum of compound **5** displayed negative and positive Cotton effects at 292 and 319 nm, respectively, which suggested the absolute stereochemistry of C-2 to be *R* (Gaffield, 1970). Accordingly, the new compound **5** was structurally characterized as (2*R*,3*R*)-7-methoxy-3,5,8-trihydroxyflavanone.

The results of the present study have shown that B-ring unsubstituted flavonoids are the major components of the leaves of *M. calabura* collected in Peru. This is in sharp contrast to a previous phytochemical study performed in our laboratory on the roots of *M. calabura* collected in the Philippines, wherein highly substituted ring-B flavans, flavones, and biflavans were characterized (Kaneda et al., 1991). In fact, only one compound, (2*S*)-5′-hydroxy-7,8,3′,4′-tetramethoxyflavan, was identified in both the Philippine and Peruvian samples of *M. calabura*. This may represent a geographical difference, and/or a difference in site-specific accumulation of different metabolites.

With the exception of lupenone (for which the solubility was found to be too low in DMSO, the solvent used), all isolates obtained in the current study were evaluated for potential to induce QR (Gerhäuser et al., 1997; Chang et al., 1997). The data obtained for active compounds are summarized in Table 2. The new flavanone **5** showed significant activity (CD 15.8 μM) in the QR induction assay. However, among the five isolated flavanones (**1**–**5**), the most potent QR induction activity was demonstrated by (2*S*)-5-hydroxy-7-methoxyflavanone (pinostrobin, **4**). The CD value of pinostrobin (**4**, $<$0.56 μM) was almost the same as that of sulforaphane (0.43 μM), the positive control substance used in this study. Furthermore, the chemoprevention index

Table 2
Quinone reductase (QR)-inducing activity by compounds **4**–**6** and **10**–**13**

Compound	CD[a]		IC_{50}[b]		CI[c]
	μg/ml	μM	μg/ml	μM	
4	<0.15	<0.56	>20	>74	>132
5	4.77	15.8	>20	>66.2	>4.2
6	5.22	17.4	15.8	52.7	3.0
10	0.7	2.9	>5	>20.8	>7.2
11	1.4	5.5	>5	>19.5	>3.5
12	1.7	5.7	>20	67.1	>11.8
13	1.42	4.6	18.6	59.6	13.0
Sulforaphane[d]	0.08	0.43	1.95	11.0	25.0

[a] Concentration required to double QR induction activity; test compounds were considered as inactive when the CD value was >5 μg/ml.
[b] Concentration required to inhibit cell growth by 50%.
[c] Chemopreventive index (CI) = IC_{50}/CD.
[d] Sulforaphane (Kennelly et al., 1997; Misico et al., 2002) was used as positive control substance.

(CI) value of this flavanone (**4**, >132) was even higher than that (25.0) of sulforaphane. A similar bioassay result was obtained for the racemic mixture form of pinostrobin, (±)-5-hydroxy-7-methoxyflavanone, which was isolated from the roots of *Renealmia nicolaioides* in our recent work (Gu et al., 2002). Compound **4** isolated in the present investigation was found to be optically pure by comparing the specific rotation value obtained in this study (−57.5°; *c* 0.80, $CHCl_3$) with the value reported for synthetic pinostrobin (−48°; *c* 1.0, $CHCl_3$) (Hodgetts, 2001).

Among the 12 isolated flavones from *M. calabura*, only compound **6** was found to be slightly active with a CD value of 17.4 μM. Two chalcones (**10** and **11**) and two isoflavonoids (**12** and **13**) exhibited more potent quinone reductase-inducing activity (Table 2), with CD values in the range of 2.9–5.7 μM. Compound **10** (2′,4′-dihydroxychalcone) was previously isolated from the leaves and stems of *M. calabura* collected in Thailand by our group, and this compound was previously found to be cytotoxic against a small panel of human and murine tumor cell lines (Nshimo et al., 1993). The other chalcone, 4,2′,4′-trihydroxychalcone (isoliquiritigenin, **11**), was recently isolated from the seeds of *Dipteryx odorata* (Tonka bean) as a potent QR-inducing principle (CD 3.8 μM) (Jang et al., in press), and then found to significantly inhibit carcinogen-induced preneoplastic lesion formation (76% at 10 μg/ml) in a mouse mammary organ culture assay (Jang et al., in press). As shown previously, isoliquiritigenin (**11**) prevents skin tumor promotion induced by 7-bromomethylbenz[*a*]anthracene with 7,12-dimethylbenz[*a*]anthracene-initiated mice (Yamamoto et al., 1991), inhibits azoxymethane (AOM)-induced murine colon carcinogenesis and AOM-induced murine colon aberrant crypt focus formation (Baba et al., 2002), suppresses metastasis, and prevents severe 5-fluorouracil-induced leukocytopenia in a pulmonary metastasis model of mouse renal cell carcinoma (Yamazaki et al., 2002). Accordingly, pinostrobin (**4**) and isoliquiritigenin (**11**) are considered worthy of further evaluation in additional assays germane to cancer chemoprevention.

3. Experimental

3.1. General

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter. The UV spectrum was obtained with a Beckman DU-7 spectrometer. The IR spectrum was run on an ATI Mattson Genesis Series FT-IR spectrophotometer. CD measurements were performed using a JASCO-710 CD spectropolarimeter. NMR spectral data were recorded at room temperature on a Bruker Avance DPX-300, NCM-360, or DRX-500 MHz spectrometer with tetramethylsilane (TMS) as internal standard. Standard pulse sequences were employed for the measurement of 2D NMR spectra (^1H–^1H COSY, HMQC, HMBC, and NOESY). FABMS was obtained on a VG 7070E-HF sector-field mass spectrometer, and EIMS, CIMS, and HRCIMS on a Finnigan/MAT 90/95 sector-field mass spectrometer. CC was carried out with Si gel G (Merck, 70–230 or 230–400 mesh). Analytical TLC was performed on 250 μm thickness Merck Si gel 60 F_{254} aluminum plates, while prep TLC was carried out on 500 or 1000 μm thickness (20×20 cm) Merck Si gel 60 F_{254} glass plates.

3.2. Plant material

The leaves of *Muntingia calabura* were collected (collection number 229) along a river bank, 10° 04′ S and 71° 06′ W, in Colombiana, River Curanja, district of Purus, province of Purus, Peru, in October 1997. The collection was performed at 325 m above sea level. A voucher specimen (P2856) has been deposited at the University of Illinois Pharmacognosy Field Station, Downers Grove, IL, USA.

3.3. Quinone reductase induction assay

For the evaluation of plants extracts, fractions, and pure isolates as inducers of QR, cultured mouse Hepa 1c1c7 cells were used as described previously (Chang et al., 1997; Misico et al., 2002). Enzyme activity was expressed as CD, the concentration required to double the specific activity of QR. IC_{50} (half-maximal inhibitory concentration of cell viability) and CI (chemoprevention index, IC_{50}/CD) values were also determined.

3.4. Extraction and isolation

The dried and milled leaves (1 kg) were extracted by maceration with MeOH (3×5 l) at room temperature, for two days each. After filtration and evaporation of the solvent under reduced pressure, the combined crude methanolic extract was suspended in H_2O (500 ml) to afford an aqueous MeOH solution (~95%), then partitioned in turn with petroleum ether (3×600 ml) and EtOAc (3×700 ml), to afford dried petroleum ether- (21 g), EtOAc- (52 g), and H_2O-soluble (56 g) extracts. The EtOAc-soluble extract showed significant quinone reductase-inducing activity (CD < 2.5 μg/ml and IC_{50} > 10 μg/ml).

The EtOAc-soluble extract was subjected to Si gel column chromatography (9×55 cm, 1.2 kg 70–230 mesh Si gel), and eluted with gradient mixtures of $CHCl_3$–MeOH (from 30:1 to 1:1), to afford 12 fractions (F01-F12), which were evaluated in the QR induction assay. The CD (μg/ml) values of F01–F12 were 6.5, >10, <2.5, <2.5, 6.5, >10, >10, >10, >10, >10, >10, and >10, respectively, and the IC_{50} values for all these 12 fractions were over 20 μg/ml. Thus, the most active fractions, F03 and F04, were chosen for further detailed purification.

The third fraction (F03), eluted with $CHCl_3$–MeOH (30:1), was fractionated over a further Si gel column (5.8×65 cm), with gradient mixtures of petroleum ether–acetone (from 8:1 to 1:1) as solvent systems, to afford six subfractions (F0301-F0306). F0302 (eluted with petroleum ether–acetone, 6:1) was further purified by a Si gel column (2.0×45 cm), using $CHCl_3$–MeOH (50:1), to give, in order of polarity, lupenone (30 mg), **2** (158 mg), 7-hydroxyflavone (9 mg), and a further subfraction, F030204. This subfraction was then purified by preparative TLC (20×20 cm, 500 μmm), developed with petroleum ether–acetone (5:1), yielding compound **14** (12 mg, R_f = 0.55) and 5,7-dihydroxyflavone (18 mg, R_f = 0.50). Subfraction F0303 (eluted with petroleum ether-acetone, 5:1) was then separated over a Si gel column (3.8×65 cm) with *n*-hexane-acetone (5:1×2:1), to afford an additional amount of **2** (78 mg), and 5-hydroxy-3,7,8-trimethoxyflavone (26 mg), (2*S*)-5′-hydroxy-7,8,3′,4′-tetramethoxyflavan (48 mg) and compound **3** (96 mg). Compound **10** (25 mg) was obtained as a yellowish amorphous powder from a n-hexane-EtOAc (~2:1) solution of subfraction F0303 (eluted with petroleum ether-acetone, 5:1). Further purification of the mother liquor of this subfraction was carried out over a Si gel column (2.0×45 cm), eluted with $CHCl_3$–acetone (10:1 to 5:2), and yielded an additional quantity of compound **10** (32 mg) and compounds **1** (62 mg), **4** (17 mg) and **13** (28 mg). Subfraction F0305 (eluted with petroleum ether-acetone, 2:1) was subjected to Si gel column chromatography (2.8×55 cm) and separated with gradient mixtures of $CHCl_3$–MeOH (from 30:1 to 10:1), to afford the pure compounds 3-methoxy-5,7,4′-trihydroxyflavone (16 mg) and **11** (23 mg).

Fraction F04, eluted with $CHCl_3$–MeOH (25:1), was subjected to Si gel column chromatography (2.8×55 cm), using petroleum ether–acetone mixtures of increasing polarity (from 8:1 to 1:1) as solvents, to afford five subfractions (F0401–F0405). Subfraction F0402 (eluted with petroleum ether-acetone, 4:1) was passed over a Si gel column (2.0×45 cm), by elution with *n*-hexane–EtOAc (from 5:1 to 2:1), and yielded 3,3′-dimethoxy-5,7,4′-trihydroxyflavone (11 mg) and 2α,3β-dihydroxyolean-12-en-28-oic acid (8 mg), and an additional quantity of (2*S*)-5′-hydroxy-7,8,3′,4′-tetramethoxyflavan (21 mg). F0403 (eluted with petroleum ether–acetone, 3:1) was subjected to purification over a Sephadex LH-20 column (2.8×55 cm) and eluted with pure MeOH, to afford compounds **5** (18 mg), 3,5-dihydroxy-7,4′-dimethoxyflavone (96 mg) and **8** (27 mg), and a mixture, F040304. This subfraction (F040304) was then purified by preparative TLC (1000 μm layers), developed twice with $CHCl_3$–MeOH (30:1), to provide pure compounds **6** (8 mg; R_f = 0.65) and **7** (15 mg; R_f = 0.63). F0404 (eluted with petroleum ether-acetone, 2:1) was purified over a further Si gel column (2.8×55 cm) and separated with $CHCl_3$–acetone (from 4:1 to 1:1), and afforded in turn pure compounds **12** (5 mg), 3,8-dimethoxy-5,7,4′-trihydroxyflavone (15 mg), **9** (158 mg) and 3,4,5-trihydroxybenzoic acid (90 mg).

3.5. (2S)-7-Hydroxyflavanone (1)

$[\alpha]_D^{23}$ −85.3° (*c* 1.2, MeOH); CD (MeOH; 20 °C; Δε) nm: 302 (−18,054), 330 (+11,800); ^{1}H (Tanrisever et al., 1987) and ^{13}C NMR (Hsieh et al., 1998) spectral data consistent with literature values.

3.6. (2S)-5,7-Dihydroxyflavanone (2)

$[\alpha]_D^{23}$ −58.5° (*c* 0.91, MeOH); CD (MeOH; 20 °C; Δε) nm: 287 (−845,500), 328 (+222,375); ^{1}H (Hsieh et al., 1998) and ^{13}C NMR (Ichino et al., 1988) spectral data consistent with literature values.

3.7. (2R,3R)-3,5,7-Trihydroxyflavanone (3)

$[\alpha]_D^{23}$ +9.0° (*c* 0.40, $CHCl_3$ + MeOH, 1:1); CD (MeOH; 20 °C; Δε) nm: 291 (−78,365), 320 (+26,738); ^{1}H and ^{13}C NMR (Kuroyanagi et al., 1982) spectral data consistent with literature values.

3.8. (2S)-5-Hydroxy-7-methoxyflavanone (4)

$[\alpha]_D^{23}$ −57.5° (*c* 0.80, $CHCl_3$); CD (MeOH; 20 °C; Δε) nm: 288 (−283,540), 331 (+70,500); ^{1}H (Ichino et al., 1988) and ^{13}C NMR (González et al., 1989) spectral data consistent with literature values.

3.9. (2R,3R)-7-Methoxy-3,5,8-trihydroxyflavanone (**5**)

Yellowish amorphous powder, mp 226–228 °C; $[\alpha]_D^{23}$ +30.0° (*c* 0.25, $CHCl_3$ + MeOH, 1:1); UV (MeOH) λ_{max} (log ε) 290 (3.82), 321 (3.94) nm; IR ν_{max} (film) cm^{-1}: 3327, 1640, 1467, 1257, 1179, 1087; CD (MeOH; 20 °C; $\Delta\varepsilon$) nm: 292 (−194,195), 319 (+60,949); EIMS *m*/*z* (rel. int.): 302 $[M]^+$ (45), 285 (3), 183 (100), 156 (16), 139 (19), 136 (21), 120 (5), 111 (9), 91 (16), 69 (9); HRCIMS *m*/*z*: 303.0866 $[M+H]^+$ (calc. for $C_{16}H_{15}O_6$, 303.0869); ^1H NMR data, see Table 1.

3.10. 2′,4′-Dihydroxydihydrochalcone (**14**)

^1H NMR spectral data (in acetone-d_6) consistent with literature (Jain and Mehta, 1985) values; ^{13}C NMR data δ_H 30.3 (C-β, *t*), 39.7 (C-α, *t*), 103.5 (C-3, *d*), 107.9 (C-5, *d*), 113.7 (C-1, *s*), 126.3 (C-4′, *d*), 128.4 (C-2′ and C-6′, *d*), 128.6 (C-3′ and C-5′, *d*), 132.2 (C-6, *d*), 140.8 (C-1′, *s*), 162.8 (C-2, *s*), 165.1 (C-4, *s*), 203.7 (C=O, *s*).

Acknowledgements

This work was supported by Program Project P01 CA48112 funded by the National Cancer Institute, NIH, Bethesda, MD. We are grateful to Drs. J. A. (Art) Anderson, Research Resources Center, University of Illinois at Chicago, and K. Fagerquist, Mass Spectrometry Facility, Department of Chemistry, University of Minnesota, Minneapolis, MN, for mass spectral data.

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