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Potential cancer chemopreventive constituents of the leaves of *Macaranga triloba*

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Abstract

Activity-guided fractionation of the leaves of *Macaranga triloba,* using an in vitro bioassay based on the inhibition of cyclooxygenase-2, resulted in the isolation of a rotenoid, 4,5-dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol (**1**), as well as 12 known compounds, (+)-clovan-2β,9α-diol, ferulic acid, 3,7,3′,4′-tetramethylquercetin, 3,7,3′-trimethylquercetin, 3,7-dimethylquercetin, abscisic acid, 1β,6α-dihydroxy-4(15)-eudesmene, 3β-hydroxy-24-ethylcholest-5-en-7-one, loliolide, scopoletin, taraxerol, and 3-*epi*-taraxerol. The structure of compound **1** was determined using spectroscopic methods. All isolates were evaluated for their potential to inhibit cyclooxygenases-1 and -2 by measuring PGE_2 production, and to induce quinone reductase in cultured Hepa 1c1c7 mouse hepatoma cells.

Keywords: *Macaranga triloba*; Euphorbiaceae; Flavonoids; 4,5-Dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol; Cyclooxygenases-1 and -2; Quinone reductase

1. Introduction

The genus *Macaranga* (Euphorbiaceae) consists of approximately 250 species that occur in the tropics of the Old World (Whitmore, 1969, 1975) with the center of distribution being tropical Asia and the Pacific (Whitmore, 1969, 1975; Fiala, 1996). This genus is known for a wide range of mutualistic associations with ants, ranging from facultative to strictly obligate relationships (Fiala et al., 1989; Fiala and Maschwitz, 1990). The leaves of *Macaranga triloba* (Bl.) Muell. Arg. (Euphorbiaceae) have been used to treat stomachache and skin itches in Indonesia and Malaysia (Ahmad and Holdsworth, 1994; Grosvenor et al., 1995). A previous phytochemical investigation on this plant has resulted in the isolation of a flavonoid, vitexin (Vinh et al., 2002). The triterpene constituents of the epicuticular wax blooms on the stems of *M. triloba* have been analyzed by GC-MS (Markstadter et al., 2000).

As part of our continuing search for novel, plant-derived cancer chemopreventive agents (Pezzuto et al., 1999; Kinghorn et al., 2003), the leaves of *M. triloba* were chosen for detailed investigation since the EtOAc-soluble extract exhibited selective inhibitory activity against cyclooxygenase-2 (COX-2). The inhibition of COX-2 as an important mechanism for cancer chemoprevention has been supported by epidemiological and experimental evidence (Dannenberg et al., 2001). Furthermore, the selective inhibition of COX-2, the inducible form of the enzyme, is believed to be more pertinent than the nonselective inhibition of both COX-1 and -2 (Bombardier et al., 2000). In the present work, activity-guided fractionation of the EtOAc-soluble extract of the leaves of *Macaranga triloba,* using an in vitro activity-guided

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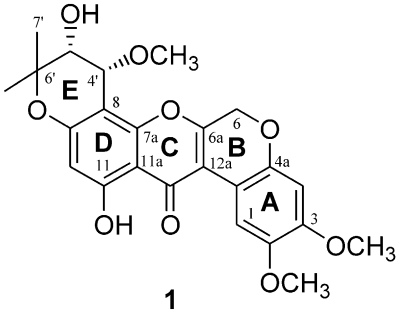
fractionation procedure based on the inhibition of COX-2, resulted in the isolation of a new rotenoid, 4,5-dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol (**1**), as well as 12 known compounds. Three of the isolates (**4**–**6**) are structurally related to known flavonoid quinone reductase (QR) inducers (Song et al., 1999; Jang et al., 2003; Su et al., 2003). QR is a phase II detoxifying enzyme and its induction is considered to be an important cancer chemopreventive mechanism at the initiation stage (Song et al., 1999). Accordingly, all pure isolates from the present investigation were evaluated for potential QR induction in the Hepa 1c1c7 mouse hepatoma cell line. The structure determination of compound **1**, as well as the biological evaluation of all isolates in three separate in vitro assays [COX-1 and COX-2 inhibition, and QR induction] are described herein.



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2. Results and discussion

Fractionation of an EtOAc-soluble partition of the MeOH extract of the leaves of *M. triloba* utilizing a COX-2 inhibition assay to monitor fractionation, led to the isolation and characterization of a new rotenoid, 4,5-dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol (**1**), along with 12 known compounds, (+)-clovan-2β,9α-diol (**2**) (Jarvis et al., 1986), ferulic acid (**3**) (Young et al., 1992), 3,7,3′,4′-tetramethylquercetin (**4**) (Nakashima et al., 1973), 3,7,3′-trimethylquercetin (**5**) (Valesi et al., 1972), 3,7-dimethylquercetin (**6**) (Wang et al., 1989), abscisic acid (Ferreres et al., 1996), 1β,6α-dihydroxy-4(15)-eudesmene (Kamel, 1995), 3β-hydroxy-24-ethylcholest-5-en-7-one (Notaro et al., 1992), loliolide (Tanaka and Matsunaga, 1989), scopoletin (Kwon et al., 2002), taraxerol (Ray et al., 1975), and 3-*epi*-taraxerol (Ray et al., 1975). The known compounds were identified by physical (mp, $[\alpha]_D$) and spectroscopic data measurement (MS, ^{1}H NMR, ^{13}C NMR and 2D NMR) and by comparison with published values. Except for taraxerol and 3-*epi*-taraxerol (Markstadter et al., 2000), all these isolates of previously known structure have not been reported from this plant.

Compound **1** was obtained as a colorless solid and gave a protonated molecular ion M^+ at *m*/*z* 456.1409 by HREIMS, consistent with an elemental formula of $C_{24}H_{24}O_9$. The ^{1}H NMR spectrum of **1** (Table 1) showed resonances for a dioxygenated dimethylchromene moiety (ring E) (Fang and Casida, 1999) at δ_H 4.41 (1H, *d*, $J = 3.5$ Hz, H-4′), δ_H 3.87 (1H, *d*, $J = 3.5$ Hz, H-5′), δ_H 1.48 (3H, *s*, H-7′) and δ_H 1.44 (3H, *s*, H-8′). After addition of D_2O (see Section 3; in acetone-d_6), the signal for H-5′ (δ_H 3.98, 1H, *dd*, $J = 5.2$ and 3.1 Hz) of **1** collapsed to a sharp doublet ($J = 3.1$ Hz), with the disappearance of the signals at δ_H 3.98 (*br d*, $J = 7.8$ Hz) and at δ_H 12.94 (1H, *s*), indicating the presence of hydroxyl groups at C-11 and C-5′. Three one-proton singlets for an aromatic ring at δ_H 8.28 (H-1), δ_H 6.61 (H-4), and δ_H 6.29 (H-10), one two-proton singlet for an oxygenated methylene at δ_H 5.06 (H-6), and three three-proton singlets for methoxyl groups at δ_H 3.95 (OCH_3-2), δ_H 3.89 (OCH_3-3), and δ_H 3.64 (OCH_3-4′) were also observed in the ^{1}H NMR spectrum of **1**. The ^{13}C NMR and DEPT experiments (Table 1) with **1** showed the presence of signals for five methine groups as well as one methylene group (δ 65.1) bearing an oxygen atom, three methoxyl groups (δ 57.9, δ 56.7 and δ 56.2), two methyl groups (δ 24.0 and δ 23.7), and 13 quaternary carbons including a conjugated ketone (δ 179.5, C-12). Comparison of the above data with those in the literature (Lin and Kuo, 1995) indicated that the structure of **1** is related to that of a rotenoid, 6a,12a-dehydro-α-toxicarol, except for the presence of a dioxygenated dimethylchromene moiety in **1**. This was confirmed by observed correlations in the 2D NMR spectra (^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) of **1**, as summarized in the following paragraph.

The HMBC correlations (Fig. 1) for H-1/C-2, C-3, C-1a, C-4a and C-12a; H-10/C-8, C-9, C-11 and C-11a; H-6/C-4a, C-6a, and C-12a; H-4′/C-8, C-9, C-7a, C-6′ and C-4′-OMe; OMe-2/C-2; and OMe-3/C-3 confirmed

Table 1
NMR spectrum data for compound **1** (in $CDCl_3$–CD_3OD 2:1)[a]

Position	δ_C	δ_H multiplicity (*J*, Hz)	Position	δ_C	δ_H multiplicity (*J*, Hz)
1	110.6 *d*	8.28 *s*	11	161.9 *s*	
1a	110.0 *s*		11a	107.1 *s*	
2	144.4 *s*		12	179.5 *s*	
3	149.7 *s*		12a	111.5 *s*	
4	101.0 *d*	6.61 *s*	4′	74.9 *d*	4.41 *d* (3.5)
4a	146.9 *s*		5′	70.4 *d*	3.87 *d* (3.5)
6	65.1 *t*	5.06 *s*	6′	79.4 *s*	
6a	157.4 *s*		7′	24.0 *q*	1.48 *s*
7a	156.2 *s*		8′	23.7 *q*	1.44 *s*
8	100.5 *s*		OCH_3-2	56.7 *q*	3.95 *s*
9	159.5 *s*		OCH_3-3	56.2 *q*	3.89 *s*
10	100.8 *d*	6.29 *s*	OCH_3-4′	57.9 *q*	3.64 *s*

[a] Run at 500 MHz for ^{1}H (*J* values in parentheses) and 125 MHz for ^{13}C. Assignments were based on COSY, HMBC, and NOESY experiments.

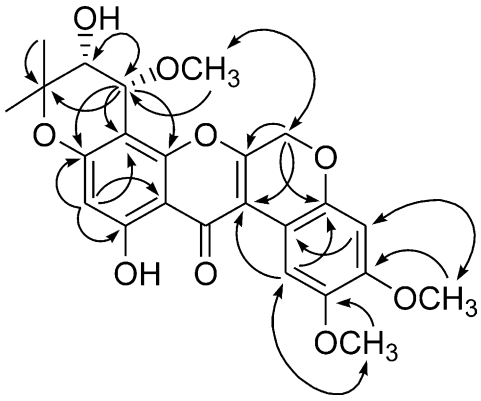

Fig. 1. Selected correlations observed in the HMBC (→) and NOESY (↔) NMR spectra of **1**.

the assignments of all proton and carbon resonances in **1** and the locations of the methoxyl and hydroxyl groups. Although their absolute stereochemistry could not be ascertained, H-4′ and H-5′ were shown to be *cis*-oriented based on the observed coupling constant ($J_{4',5'} = 3.5$ Hz) and a NOE correlation (Fig. 1) between these two protons (Fang and Casida, 1999). Therefore, the relative stereochemistry of this new rotenoid (**1**) could be elucidated as 4,5-dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol. There is only a single report on the isolation of a rotenoid from the genus *Macaranga* (Sultana and Ilyas, 1987).

All isolates obtained in this study were evaluated for their potential to inhibit COX-1 and -2 and to induce QR (Table 2). Compound **1** was inactive in all assays used in this investigation. The known compounds (+)-clovan-2β,9α-diol (**2**) and ferulic acid (**3**) showed moderate inhibitory activities against COX-2 (IC_{50} 46.6 μg/ml) and COX-1 (IC_{50} 33.7 μg/ml), respectively. In our recent investigations, several flavanones, flavones, isoflavones, and chalcones have been found to exhibit significant in vitro QR induction activity (Jang et al., 2003; Su et al., 2003). Flavones with a methoxyl group at position C-8 were found to be inactive or less active than chalcones and isoflavones in the QR induction assay (Su et al., 2003). In the present study, three quercetin derivatives (**4**–**6**), which do not have any methoxyl group at position C-8, exhibited potent QR activity with CD values ranging from 0.5 to 0.8 μg/ml (Table 2). However, further investigations are needed to determine whether the QR induction potency of the flavones **4**–**6** is due to the absence of a methoxyl group at position C-8. Compounds **4** (3,7,3′,4′-tetramethylquercetin), found in *Citrus sinensis* L. (Manthey and Guthrie, 2002), and **6** (3,7-dimethylquercetin), found in rosemary honey (from *Rosmarinus officinalis* L.) (Gil et al., 1995), appear promising as QR inducing agents, since they are potent yet not appreciably cytotoxic for the host cells, although their analogue compound, **5**, was considerably cytotoxic to Hepa 1c1c7 cells at the dose tested. Compounds **4** and **6**, in being known constituents of edible plants and with promising activity in the QR assay, are worthy of additional biological evaluation for their potential as cancer chemopreventive agents.

Table 2
Inhibitory activities against cyclooxygenase-1 (COX-1) and -2 (COX-2), and potential to induce quinone reductase (QR) of compounds **2**–**6** isolated from *M. triloba*[a]

Compound	COX [IC_{50} (μg/ml)]		QR[b]		
	COX-1	COX-2	CD (μg/ml)	IC_{50} (μg/ml)	CI
2	>100	46.6	>10	>10	ND[c]
3	33.7	>100	>10	>10	ND[c]
4	>100	>100	0.8	41.8	52.3
5	>100	>100	0.5	1.3	2.6
6	>100	>100	0.5	38.3	76.6
trans-Resveratrol[d]	0.25	0.30			
4′-Bromoflavone[e]			0.004	>49.8	>12,450

[a] The new compound **1** and the known compounds, abscisic acid, 1β,6α-dihydroxy-4(15)-eudesmene, 3β-hydroxy-24-ethylcholest-5-en-7-one, loliolide, scopoletin, taraxerol, and 3-*epi*-taraxerol were inactive (IC_{50} >100 μg/ml for COX assays and CD >10 μg/ml for QR assay) in the COX-1, COX-2, and QR assays.

[b] CD, concentration required to double QR activity; IC_{50}, concentration inhibiting cell growth by 50%; CI, Chemoprevention Index (=IC_{50}/CD).

[c] Not determined since the exact CD value of compound was not accurately evaluated.

[d] *trans*-Resveratrol (Waffo-Téguo et al., 2001) was used as a positive control in the COX assays.

[e] 4′-Bromoflavone (Song et al., 1999) was used as a positive control in the QR assay.

3. Experimental

3.1. General

Melting points were determined on a Fisher-Johns melting-point apparatus and are uncorrected. Optical rotations were obtained using a Perkin-Elmer 241 polarimeter. UV spectra were recorded with a Beckman DU-7 spectrometer. IR spectra were run on an ATI Mattson Genesis Series FT-IR spectrometer. NMR experiments were conducted on Bruker DPX-300 and DRX-500 MHz spectrometers with tetramethylsilane (TMS) as internal standard. EIMS and HREIMS were obtained on a Finnigan MAT 95 sector-field mass spectrometer operating at 70 eV. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich, Milwaukee, WI) followed by charring at 110 °C for 5–10 min. Silica gel (Merck 60A, 70–230 or 200–400 mesh ASTM) and Sorbisil C_{18} reversed-phase silica gel (Sigma, St. Louis, MO) were used for cc. Preparative

TLC was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness). All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ) and distilled before use.

3.2. COX inhibition assay

Inhibition assays against both COX-1 and -2 were performed as described previously by measuring PGE_2 production (Cuendet and Pezzuto, 2000; Waffo-Téguo et al., 2001; Jang et al., 2002).

3.3. Quinone reductase induction assay

For the evaluation of the pure isolates as inducers of quinone reductase (QR), cultured mouse Hepa 1c1c7 cells were used as described previously (Gerhäuser et al., 1997; Song et al., 1999).

3.4. Plant material

The leaves of *Macaranga triloba* (Bl.) Muell. Arg. (Euphorbiaceae) were collected in Kintap, West Kalimantan, Indonesia, in August 1994 by K.K., H.H.S.F., N.R.F. and L.B.S.K. Voucher specimens (number K002) have been deposited at the Herbarium Bogoriense, Bogor, Indonesia and at the Field Museum of Natural History, Chicago, IL, USA.

3.5. Extraction and Isolation

The dried and milled plant material (920 g) was extracted with MeOH (3×4 l) by maceration. The extracts were combined and concentrated in vacuo at 40 °C. The concentrated extract was suspended in 90% MeOH and then partitioned with petroleum ether (3×2 l) to afford a petroleum ether-soluble syrup (12.3 g) on drying. Next, the aq MeOH extract was concentrated and suspended in H_2O (2 l) and partitioned again with EtOAc (3×2 l) to give an EtOAc-soluble extract (33.5 g) and an aqueous residue (82.2 g). The EtOAc-soluble extract showed significant COX-2 inhibition activity (55% inhibition at 10 μg/ml), but was inactive in the COX-1 (<50% inhibition at 10 μg/ml) and QR induction (CD value >10 μg/ml) assays.

The EtOAc-soluble extract (D002) was subjected to silica gel chromatography (7×40 cm, 70–230 mesh) using a $CHCl_3$–MeOH gradient (from 19:1 to 0:1 v/v) to yield 12 pooled fractions (F004–F015). Of these, fractions F007, F009 and F010 showed the most potent COX-2 inhibitory activities (80, 55 and 51% inhibition at 10 μg/ml, respectively). Separation of fraction F007 (eluted with $CHCl_3$–MeOH, 19:1 v/v, 1.01 g) on silica gel (2.5×25 cm, 230–400 mesh) with gradient mixtures of petroleum ether-EtOAc (from 4:1 to 0:1 v/v) afforded fractions F016-F026. 3β-Hydroxy-24-ethylcholest-5-en-7-one (6.5 mg, 0.00071%) was isolated from fraction F016, eluted with petroleum ether–EtOAc (4:1 v/v), by recrystallization from MeOH. Fractions F019 and F020 [eluted with petroleum ether–EtOAc (3:1 v/v), 350 mg] were combined, and then further purified by a silica gel column (2×25 cm, 230–400 mesh) with $CHCl_3$–MeCN (49:1 v/v), to give, in order of polarity, 3,7,3′,4′-tetramethylquercetin (**4**, 1.2 mg, 0.00013%), 3,7,3′-trimethylquercetin (**5**, 3.3 mg, 0.00036%), 3,7-dimethylquercetin (**6**, 8.0 mg, 0.00087%), and 1β,6α-dihydroxy-4(15)-eudesmene (2.8 mg, 0.00030%). The triterpenoids, 3-*epi*-taraxerol (115 mg, 0.0125%) and taraxerol (176 mg, 0.0191%) were purified from fractions F004 and F005, respectively, by recrystallization from petroleum ether.

The two other active fractions, F009 and F010 [eluted with $CHCl_3$–MeOH (19:1 v/v), 2.0 g] were combined, and then subjected to silica gel cc (4×34 cm, 70–230 mesh) with petroleum ether–EtOAc (4:1→0:1, v/v) to afford fractions F027-F037. Fraction F031 [eluted with petroleum ether–EtOAc (3:2 v/v), 450 mg] was further purified by reversed-phase low-pressure liquid chromatography over C_{18} silica gel (gradient from 60% MeOH in H_2O to 100% MeOH as the solvent), resulting in the isolation of loliolide (16 mg, 0.0017%) and ferulic acid (**3**, 34 mg, 0.0037%). Fractions F034 and F035 [eluted with petroleum ether-EtOAc (1:3 v/v), 720 mg] were combined, and then passed over a column containing Sephadex LH-20 (Sigma, St. Louis, MO) using $CHCl_3$–MeOH (1:1 v/v) for elution, yielding, in turn, scopoletin (1.0 mg, 0.00011%), abscisic acid (4.3 mg, 0.00047%), (+)-clovan-2β,9α-diol (**2**, 3.8 mg, 0.00041%), and the new compound **1** (1.2 mg, 0.00013%).

3.5.1. 4,5-Dihydro-5′α-hydroxy-4′α-methoxy-6a,12a-dehydro-α-toxicarol (*1*)

Yellow needles, mp 215–218 °C; $[\alpha]_D^{20}$ +0.1° (*c* 0.1, $CHCl_3$–MeOH 1:1); UV λ_{max} EtOH nm (log ϵ): 207 (4.61), 277 (4.39), 302 (4.09), 320 (4.05); IR ν_{max} NaCl cm^{1}: 3485, 2924, 2852, 1736, 1657, 1613, 1582, 1510, 1453, 1343, 1288, 1154, 1039, 753; ^{1}H NMR data (in acetone-d_6, 300 MHz): δ_H 12.94 (1H, *s*, OH-11), 8.31 (1H, *s*, H-1), 6.65 (1H, *s*, H-4), 6.18 (1H, *s*, H-10), 5.19 (2H, *d*, *J* = 3.4 Hz, H-6), 4.54 (1H, *d*, *J* = 5.4 Hz, OH-5′), 4.45 (1H, *d*, *J* = 3.1 Hz, H-4′), 3.98 (1H, *dd*, *J* = 5.2 and 3.1 Hz, H-5′), 3.84 (3H, *s*, OCH_3-2′ or 3′), 3.83 (3H, *s*, OCH_3-2′ or 3′), 3.63 (3H, *s*, OCH_3-4′), 1.45 (3H, *s*, H-7′ or 8′), 1.42 (3H, *s*, H-7′ or 8′) [on addition of D_2O, the OH-11 and OH-5′ signals disappeared and the H-5′ signal collapsed to sharp doublet (*J* = 3.1 Hz)]; EIMS *m*/*z* (rel. int.): 456 ($[M]^+$ 100), 424 (46), 385 (23), 369 (23), 191 (4); HREIMS *m*/*z*: 456.1409 ($[M]^+$ calc. for $C_{24}H_{24}O_9$, 456.1420); ^{1}H and ^{13}C NMR data (in $CDCl_3$–CD_3OD 2:1), see Table 1.

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