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Isolation and absolute stereochemistry of coussaric acid, a new bioactive triterpenoid from the stems of *Coussarea brevicaulis*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Coussaric acid (**1**), a triterpenoid based on an ursane skeleton, and an oleanane-type triterpene acid, 3-*epi*-spathodic acid (**2**), as well as four known compounds, barbinervic acid, scutellaric acid, stigmasterol and stigmasterol glucoside, have been isolated from an EtOAc-soluble extract of the stems of *Coussarea brevicaulis*. The structures of compounds **1** and **2** were elucidated on the basis of spectroscopic investigation, and single-crystal X-ray crystallography was used to confirm the structure of **1**. The absolute stereochemistry of **1** was established by chemical transformations and by the Mosher ester procedure. The potential of the isolates and chemical transformation products to induce quinone reductase was evaluated in mouse Hepa lclc7 hepatoma cells.

Keywords: *Coussarea brevicaulis*; Rubiaceae; Coussaric acid; Single-crystal X-ray analysis; Chemical transformation; Mosher ester method; Quinone reductase induction assay

1. Introduction

Cancer chemoprevention is a strategy for reducing cancer mortality and involves the prevention, delay, or reversal of cancer by the ingestion of dietary or pharmaceutical agents capable of modulating the process of carcinogenesis, in order to prevent rather than wait to treat cancers (Wattenberg, 1985; Morse and Stoner, 1993; Hong and Sporn, 1997). During our collaborative research in this area, several different assays have been used to direct the chromatographic fractionation of crude plant extracts, and a number of plant-derived potential cancer chemopreventive agents have been isolated and evaluated (Pezzuto, 1997; Kinghorn et al., 1998, 2003; Park and Pezzuto, 2002). Induction of Phase 2 drug-metabolizing enzymes, such as quinone reductase (QR), is considered an effective and sufficient 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 naturally occurring cancer chemopreventive agents, a methanolic extract of the stems of *Coussarea brevicaulis* Krause (Rubiaceae) was found to be a weak inducer of QR activity in cultured Hepa lclc7 mouse hepatoma cells (Prochaska and Santamaria, 1988; Misico et al., 2002). A literature survey revealed no previous investigations on the chemical constituents of the genus *Coussarea*. In the present communication, we report the isolation and identification of coussaric acid (**1**), a new triterpenoid based on the ursane skeleton. The structure and relative stereochemistry of this compound were proposed using

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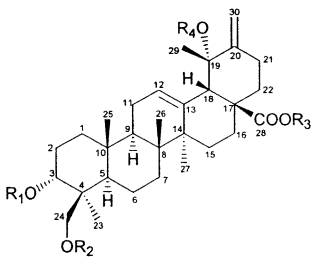

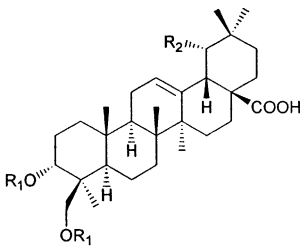

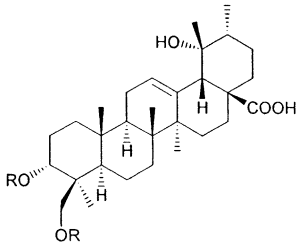

1 $R_1 = R_2 = R_3 = R_4 = H$
1a $R_1 = R_2 = Ac$ $R_3 = R_4 = H$
1b $R_1 = R_3 = R_4 = H$ $R_2 = Ac$
1c $R_1 = R_2 = R_4 = H$ $R_3 = Me$
1d $R_1 = R_2 = Ac$ $R_3 = Me$ $R_4 = H$
1e $R_1 = R_4 = H$ $R_2 = Ac$ $R_3 = Me$
1r $R_1 = R_4 = (R)$-MTPA $R_2 = Ac$ $R_3 = Me$
1s $R_1 = R_4 = (S)$-MTPA $R_2 = Ac$ $R_3 = Me$

2 $R_1 = H$ $R_2 = OH$
2a $R_1 = Ac$ $R_2 = OH$
4 $R_1 = R_2 = H$

3 R = H
3a R = Ac

spectroscopic methods, and confirmed using single-crystal X-ray crystallography. The absolute configuration was determined for the derivative **1e** (the 24-monoacetate of the methyl ester of **1**) using the Mosher ester procedure (Dale and Mosher, 1973; Sullivan et al., 1973; Ohtani et al., 1991), as carried out in a convenient manner in an NMR tube (Su et al., 2002). Also isolated in the present investigation was a second new triterpene, 3-*epi*-spathodic acid (**2**), isolated and characterized in the form of its 3,24-diacetate (**2a**), and four known compounds. These substances were evaluated in the QR induction as pure entities, and the results are described herein.

2. Results and discussion

The EtOAc-soluble extract (5.8 g) of the stems of *C. brevicaulis* was fractionated by repeated Si gel chromatography and HPLC, and led to the isolation of two triterpenoids, coussaric acid (**1**) and 3-*epi*-spathodic acid (**2**), as well as four known compounds, barbinervic acid (**3**) (Delle Monache et al., 1996), scutellaric acid (**4**) (Morota et al., 1995), stigmasterol (Ikawa et al., 1994), and stigmasterol glucoside (Backheet and Sayed, 2002). The structures of the known compounds were identified by physical and spectroscopic data measurement ($[\alpha]_D$, ^1H NMR, ^{13}C NMR, DEPT, 2D NMR, and MS) and by comparing the data obtained with those of literature values.

Compound **1** was obtained as colorless needles ($CHCl_3$–MeOH; 5:1), mp 232–233 °C, $[\alpha]_D^{23}$ +51.5° (*c* 1.0, pyridine). A molecular formula of $C_{30}H_{46}O_5$ was determined for this compound from the molecular ion peak at *m*/*z* 486.3355 $[M]^+$ (calc. for $C_{30}H_{46}O_5$, 486.3345) obtained by HREIMS, consistent with eight degrees of unsaturation. The ^1H NMR spectrum (Table 1) of compound **1** displayed the characteristic signals of five methyl groups (all singlets, δ_H 1.01, 1.12, 1.64, 1.66, 1.70), a hydroxymethyl group (δ_H 3.83, *d*, *J* = 10.8 Hz, H-24a; 4.11, *d*, *J* = 10.8 Hz, H-24b), an oxygenated methine (δ_H 4.46, *br s*, H-3), an olefinic proton (δ_H 5.46, *br s*, H-12), and an exomethylene (δ_H 4.81, *s*, H-30a; 5.01, *s*, H-30b). Consistent with the determined molecular formula and the above ^1H NMR data analysis, the ^{13}C and DEPT NMR spectra (Table 2) of compound **1** showed 30 carbon signals, including five methyls (δ_C 16.1, 17.3, 23.6, 24.0 and 27.6), an oxygenated methylene (δ_C 65.7, C-24), an oxygenated methine (δ_C 70.0, C-3), an oxygenated quaternary carbon (δ_C 73.0, C-19), two double bonds (δ_C 128.3, 139.6, C-12/C-13; δ_C 156.7, 105.3, C-20/C-30), and a carbonyl acid group (δ_C 180.3). Accordingly, compound **1** could be assigned as a pentacyclic triterpenoid containing two double bonds and a carbonyl acid group. In the HMBC spectrum of **1**, the proton signal of CH_3-23 was correlated to the carbon signals of the oxygenated methine (C-3) and the hydroxymethyl (C-24), an aliphatic methine (C-5) and quaternary carbon (C-4), and indicated that the oxygenated methine and hydroxymethyl are located at C-3 and C-24, respectively. The oxygenated quaternary carbon and the exomethylene were assigned at C-19 and C-20 based on the observed HMBC correlations from H-30a and H-30b to C-19, C-20 and C-21, and from CH_3-29 to C-18, C-19 and C-20. Generally, the carbon chemical shifts for CH_3-23 (α-configuration) are in the range of 22–25 ppm for triterpenoids possessing a β-hydroxymethyl at C-4, while the carbon chemical shifts for CH_3-24 (β-configuration) are about 12–19 ppm for triterpenoids possessing a C-4 α-hydroxymethyl (Sakakibara et al., 1984; Ikuta and Itokawa, 1988; Nakatani et al., 1989; Ngouela et al., 1990; Deepak and Handa, 1998). The ^{13}C NMR signal for CH_3-23 was assigned at δ_C 23.6 by analysis of HMQC and HMBC correlations of compound **1**, indicating the oxygenated methyl to be β-oriented. This was confirmed by the NOESY correlations from CH_3-25 to both H-24a and H-24b. When compared to

Table 1
^{1}H NMR spectral data for **1**, **1a**–**1c**, **1e**, **2a** and **3a**[a]

Position	**1**[b]	**1a**[b]	**1b**[b]	**1c**[c]	**1e**[b]	**2a**[b]	**3a**[b]
1	1.90, *m*	1.30–1.44, *m*	1.80–1.85, *m*	1.44–1.62, *m*	1.64–1.67, *m*	1.23–1.37, *m*	1.25–1.41, *m*
	1.41–1.48, *m*		1.27–1.45, *m*	1.30–1.39, *m*	1.33–1.38, *m*		
2	2.11–2.17, *m*	1.94–1.97, *m*	2.00–2.18, *m*	1.65–1.75, *m*	1.89–2.00, *m*	1.95–2.03, *m*	2.04–2.10, *m*
	1.86, *m*	1.65–1.72, *m*	1.80–1.85, *m*	1.44–1.62, *m*	1.81–1.84, *m*	1.67–1.72, *m*	1.66–1.71, *m*
3	4.46, *br s*	5.30, *br s*	4.08, *br s*	3.87, *br s*	4.07, *br s*	5.29, *br s*	5.29, *br s*
5	1.95, *br d* (12.6)	1.54–1.61, *m*	1.92, *br d* (12.0)	1.30–1.39, *m*	1.89–2.00, *m*	1.55–1.61, *m*	1.50–1.63, *m*
6	1.72–1.76, *m*	1.54–1.61, *m*	1.64–1.73, *m*	1.44–1.62, *m*	1.64–1.67, *m*	1.55–1.61, *m*	1.50–1.63, *m*
	1.56, *m*		1.27–1.45, *m*	1.30–1.39, *m*	1.47, *m*	1.23–1.37, *m*	1.25–1.41, *m*
7	1.72–1.76, *m*	1.30–1.40, *m*	1.64–1.73, *m*	1.44–1.62, *m*	1.81–1.84, *m*	1.23–1.37, *m*	1.66–1.71, *m*
	1.41–1.48, *m*		1.27–1.45, *m*	1.30–1.39, *m*	1.33–1.38, *m*		1.37–1.41, *m*
9	2.11–2.17, *m*	1.94–1.97, *m*	2.00–2.18, *m*	1.65–1.75, *m*	2.04, *m*	1.95–2.03, *m*	1.92–1.98, *m*
11	2.11–2.17, *m*; 2.06, *m*	2.06–2.18, *m*	2.00–2.18, *m*	2.00–2.04, *m*	2.09–2.17, *m*	1.95–2.03, *m*	2.00–2.07, *m*
				1.84–1.96, *m*	1.89–2.00, *m*		
12	5.64, *br s*	5.64, *br s*	5.63, *br s*	5.43, *t* (3.5)	5.52, *t* (3.3)	5.59, *br s*	5.63, *br s*
15	2.31–2.35, *m*	2.24–2.36, *m*	2.23–2.36, *m*	1.44–1.62, *m*	2.09–2.17, *m*	2.10–2.23, *m*	2.33, *td* (13.4, 4.4)
	1.33, *m*	1.30–1.44, *m*	1.27–1.45, *m*	1.05, *m*	1.22, *m*	1.23–1.37, *m*	1.25–1.41, *m*
16	3.20–3.27, *m*	3.14–3.28, *m*	3.14–3.29, *m*	2.47, *td* (13.6, 4.3)	3.19, *td* (13.5, 4.2)	2.83, *m*	3.13, *td* (13.5, 4.3)
	2.11–2.17, *m*	2.24–2.36, *m*	2.00–2.18, *m*	1.65–1.75, *m*	1.89–2.00, *m*	2.10–2.23, *m*	2.04–2.10, *m*
18	3.25, *br s*	3.26, *br s*	3.22, *br s*	2.88, *br s*	3.01, *br s*	3.64, *m*	3.07, *br s*
19						3.64, *m*	
20							1.50–1.63, *m*
21	3.17, *td* (12.7, 4.1)						
	3.14–3.28, *m*	3.14–3.29, *m*	2.68, *td* (13.0, 5.7)	3.09, *td* (13.2, 4.2)	2.10–2.13, *m*	2.04–2.10, *m*	
	2.45, *ddd* (11.9, 4.1, 4.1)	2.24–2.36, *m*	2.23–2.36, *m*	2.21, *ddd* (13.0, 5.6, 2.6)	1.89–2.00, *m*	1.23–1.37, *m*	1.25–1.41, *m*
22	2.31–2.35, *m*	2.24–2.36, *m*	2.23–2.36, *m*	1.84–1.96, *m*	2.09–2.17, *m*	2.10–2.13, *m*	2.18, *m*
	2.11–2.17, *m*	2.14–2.18, *m*	2.00–2.18, *m*	1.65–1.75, *m*	1.81–1.84, *m*	1.23–1.37, *m*	2.04–2.10, *m*
23	1.64, *s*	1.12, *s*	1.41, *s*	1.09, *s*	1.41, *s*	1.12, *s*	1.12, *s*
24	3.17, *td* (12.7, 4.1)	3.14–3.28, *m*	3.14–3.29, *m*	2.68, *td* (13.0, 5.7)	3.04, *td* (13.2, 4.2)	2.10–2.13, *m*	2.04–2.10, *m*
	2.45, *ddd* (11.9, 4.1, 4.1)	2.24–2.36, *m*	2.23–2.36, *m*	2.21, *ddd* (13.0, 5.6, 2.6)	1.89–2.00, *m*	1.23–1.37, *m*	1.25–1.41, *m*
25	1.01, *s*	0.86, *s*	0.94, *s*	0.90, *s*	0.99, *s*	0.87, *s*	0.85, *s*
26	1.12, *s*	1.06, *s*	1.09, *s*	0.70, *s*	0.89, *s*	1.03, *s*	1.07, *s*
27	1.70, *s*	1.72, *s*	1.68, *s*	1.22, *s*	1.61, *s*	1.59, *s*	1.68, *s*
29	1.66, *s*	1.68, *s*	1.65, *s*	1.41, *s*	1.58, *s*	1.13, *s*	1.47, *s*
30	5.01, *s*; 4.81, *s*	5.02, *s*; 4.83, *s*	5.01, *s*; 4.82, *s*	5.01, *s*; 4.78, *d* (1.2)	4.96, *s*; 4.79, *s*	1.19, *s*	1.13, *d* (5.1)[d]
OH-3					5.83, *br d* (3)		
OH-19	5.74, *s*	5.85, *br s*	5.79, *s*	1.55, *s*	5.82, *s*	5.96, *d* (5.4)	5.10, *s*
OMe				3.61, *s*	3.71, *s*		
3-OAc		2.10, *s*				2.00, *s*	2.07, *s*
24-OAc		2.01, *s*	2.05, *s*		2.07, *s*	2.01, *s*	2.00, *s*

[a] The data for **1e** were obtained at 500 MHz, while the data for all other compounds were obtained at 360 MHz, and TMS was used as internal standard, with signals assigned based on 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, NOESY, and ROESY) correlations.
[b] In pyridine-d_5.
[c] In $CDCl_3$.
[d] Coupling constant partially observed.

3β,24-hydroxytriterpenoids (Nakatani et al., 1989; Ngouela et al., 1990), C-3 of compound **1** showed a significant upfield chemical shift (8–10 ppm), and the signal of H-3 in **1** was displayed as a broad singlet ($W_{1/2}$ ~3 Hz) instead of a doublet of doublets (7–12 and 4–7 Hz) as for 3β-hydroxytriterpenoids (Sakakibara et al., 1984; Nakatani et al., 1989; Ngouela et al., 1990). This suggested compound **1** should possess an 3α-OH rather than the more usual 3β-OH. The structure and relative configuration of coussaric acid A (**1**) were confirmed unambiguously by X-ray diffraction analysis (Fig. 1).

Recently, the absolute stereochemistry of selected natural products was determined by a convenient Mosher ester procedure, in which the samples were treated with (*R*)- and (*S*)-MTPA-Cl in deuterated pyridine directly in NMR tubes, to afford the (*S*)- and (*R*)-MTPA esters, respectively (Su et al., 2002). Accordingly, the reactions can be conveniently monitored by running ^{1}H NMR spectra at intervals, and the NMR spectral data of the MTPA esters so produced can be acquired without purification (Su et al., 2002). In order to determine the absolute stereochemistry of compound **1** by this method, coussaric acid (**1**) was first selectively acetylated using pyridine and acetic anhydride in an ice-water bath for 10 min, to give the 3α,24-diacetate (**1a**) and the 24-monoacetate (**1b**) of **1** as a minor and a major product, respectively. However, when the 24-monoacetate (**1b**) of **1** was treated with (*R*)-

Table 2
^{13}C NMR spectral data for **1**–**3**, **1a**–**1c**, **1e**, **2a** and **3a**[a]

Position	**1**[b]	**1a**[b]	**1b**[b]	**1c**[c]	**1e**[b]	**2**[b]	**2a**[b]	**3**[b]	**3a**[b]
1	34.0 *t*	33.8 *t*	33.5 *t*	33.3 *t*[d]	33.9 *t*	33.8 *t*	33.64 *t*[d]	34.0 *t*[d]	33.7 *t*[d]
2	26.5 *t*	22.9 *t*	26.3 *t*	25.2 *t*	26.3 *t*[d]	26.5 *t*	22.9 *t*	26.5 *t*[e]	22.9 *t*
3	70.0 *d*	73.4 *d*	69.8 *d*	70.6 *d*	69.9 *d*	70.0 *d*	73.4 *d*	69.9 *d*	73.4 *d*
4	43.9 *s*	40.8 *s*	41.9 *s*	42.7 *s*	42.01 *s*[e]	43.9 *s*	40.8 *s*	44.0 *s*	40.8 *s*
5	50.2 *d*	51.3 *d*	49.9 *d*	49.6 *d*	49.9 *d*	50.3 *d*	51.4 *d*	50.2 *d*	51.3 *d*
6	19.2 *t*	18.7 *t*	18.9 *t*	18.6 *t*	19.0 *t*	19.2 *t*	18.7 *t*	19.2 *t*	18.7 *t*
7	34.2 *t*	33.8 *t*	34.2 *t*	33.2 *t*[d]	33.6 *t*	33.7 *t*	33.60 *t*[d]	34.1 *t*[d]	33.6 *t*[d]
8	40.4 *s*	40.2 *s*	40.3 *s*	39.6 *s*	40.3 *s*	40.2 *s*	40.1 *s*	40.6 *s*	40.4 *s*
9	47.8 *d*	47.7 *d*	47.7 *d*	47.2 *d*	47.7 *d*	48.4 *d*	48.3 *d*	47.8 *d*	47.7 *d*
10	37.5 *s*	37.1 *s*	37.3 *s*	36.9 *s*	37.4 *s*	37.7 *s*	37.3 *s*	37.5 *s*	37.1 *s*
11	24.2 *t*	24.1 *t*	24.1 *t*	23.7 *t*	24.1 *t*	24.4 *t*	24.2 *t*	24.3 *t*	24.1 *q*
12	128.3 *d*	128.0 *d*	128.2 *d*	129.4 *d*	128.5 *d*	123.5 *d*	123.2 *d*	128.1 *d*	127.8 *d*
13	139.6 *s*	139.6 *s*	139.6 *s*	138.0 *s*	139.1 *s*	144.8 *s*	144.9 *s*	140.0 *s*	140.0 *s*
14	42.2 *s*	42.2 *s*	42.2 *s*	41.4 *s*	42.00 *s*[e]	42.1 *s*	42.1 *s*	42.1 *s*	42.1 *s*
15	29.2 *t*	29.2 *t*	29.2 *t*	27.8 *t*	28.9 *t*	29.2 *t*	29.2 *t*[e]	29.3 *t*	29.3 *t*
16	26.8 *t*	26.7 *t*	26.8 *t*	25.5 *t*	26.4 *t*[d]	28.4 *t*	28.3 *t*	26.4 *t*[e]	26.4 *t*
17	48.4 *s*	48.3 *s*	48.3 *s*	47.9 *s*	48.6 *s*	46.1 *s*	46.0 *s*	48.3 *s*	48.3 *s*
18	55.4 *d*	55.3 *d*	55.3 *d*	54.3 *d*	55.2 *d*	44.8 *d*	44.8 *d*	54.6 *d*	54.6 *d*
19	73.0 *s*	73.0 *s*	73.0 *s*	72.6 *s*	72.9 *s*	81.2 *d*	81.3 *d*	72.7 *s*	72.7 *s*
20	156.7 *s*	156.6 *s*	156.7 *s*	152.6 *s*	156.3 *s*	35.7 *s*	35.8 *s*	42.4 *d*	42.4 *d*
21	29.0 *t*	28.9 *t*	29.0 *t*	27.5 *t*	28.7 *t*	29.1 *t*	29.1 *t*[e]	27.0 *t*	26.9 *t*
22	39.5 *t*	39.6 *t*	39.6 *t*	36.0 *t*	39.3 *t*	33.7 *t*	33.4 *t*	38.5 *t*	38.5 *t*
23	23.6 *q*	22.2 *q*	23.3 *q*	21.7 *q*	23.3 *q*	23.7 *q*	22.3 *q*	23.7 *q*	22.3 *q*
24	65.7 *t*	66.9 *t*	68.3 *t*	66.6 *t*	68.3 *t*	65.7 *t*	66.8 *t*	65.7 *t*	66.9 *t*
25	16.1 *q*	15.6 *q*	15.8 *q*	15.8 *q*	15.8 *q*	15.8 *q*	15.4 *q*	16.0 *q*	15.5 *q*
26	17.3 *q*	17.2 *q*	17.2 *q*	16.7 *q*	17.0 *q*	17.4 *q*	17.4 *q*	17.2 *q*	17.0 *q*
27	24.0 *q*	24.0 *q*	23.9 *q*	23.7 *q*	23.9 *q*	24.7 *q*	24.77 *q*	24.6 *q*	24.5 *q*
28	180.3 *s*	180.2 *s*	180.2 *s*	177.7 *s*	178.0 *s*	181.0 *s*	181.0 *s*	180.7 *s*	180.7 *s*
29	27.6 *q*	27.5 *q*	27.5 *q*	29.8 *q*	27.2 *q*	24.8 *q*	24.80 *q*	27.1 *q*	27.1 *q*
30	105.3 *t*	105.4 *t*	105.3 *t*	107.1 *t*	105.5 *t*	28.9 *q*	28.9 *q*	16.8 *q*	16.8 *q*
OMe				51.8 *q*	51.7 *q*				
3-OAc		170.4 *s*					170.4 *s*		170.4 *s*
		21.1 *q*					21.0 *q*		21.1 *q*
24-OAc		171.0 *s*	171.1 *s*		171.1 *s*		171.0 *s*		171.0 *s*
		20.7 *q*	20.9 *q*		20.8 *q*		20.7 *q*		20.7 *q*

[a] The spectrum for **1e** was obtained at 125 MHz, while all other spectra were obtained at 90 MHz, TMS was used as internal standard, and data were assigned based on the 2D NMR (^{1}H–^{1}H COSY, HMQC, HMBC, NOESY, and ROESY) correlations.
[b] In pyridine-d_5.
[c] In $CDCl_3$.
[d] Assignments may be interchangeable in the same column.
[e] Assignments may be interchangeable in the same column.

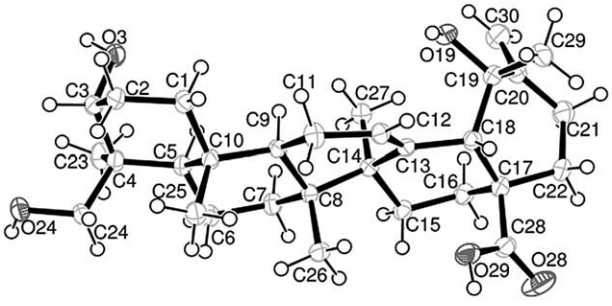

Fig. 1. ORTEP drawing of coussaric acid (**1**).

and (*S*)-MTPA-Cl using the above-mentioned method, the ^{1}H NMR spectra of the products were quite complex and the data were not clear enough to determine the absolute configuration. It is possible that the carbonyl acid group in **1b** may react with MTPA-Cl to give an anhydride, which would be easily hydrolyzed to a carbonyl acid derivative. To overcome this problem, coussaric acid (**1**) was first methylated to afford the methyl ester **1c**, which was then selectively acetylated by the method used for **1**, and the 3α,24-diacetate (**1d**) and 24-monoacetate (**1e**) of **1c** were obtained. The 24-monoacetate of the methyl ester of coussaric acid (**1e**) was used to determine the absolute stereochemistry using the above-mentioned convenient Mosher ester procedure (Su et al., 2002). In consideration of the possible steric hindrance of CH_3-23 and 4-CH_2OAc on 3-OH, a catalytic amount of *N*,*N*-dimethyl-4-aminopyridine (DMAP) was added to the reaction NMR tubes. The ^{1}H NMR spectra (Fig. 2) of the diastereomeric MTPA esters (**1r** and **1s**) of **1e** were obtained by

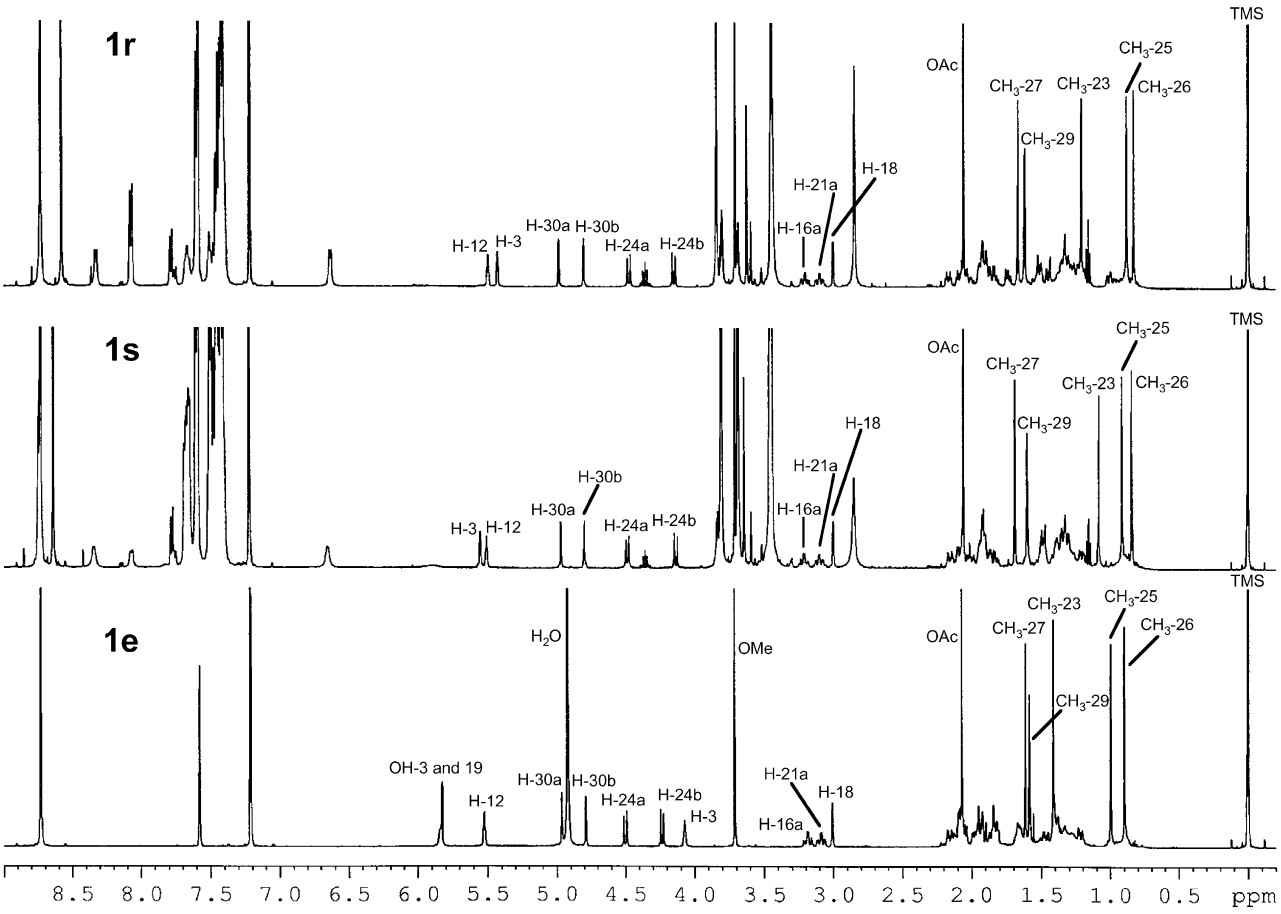

Fig. 2. ^{1}H NMR spectra of **1e**, **1s** and **1r**: all spectra were obtained in pyridine-d_5 at 500 MHz with TMS as internal standard; although strong signals of excess amount of reagents are present in **1s** and **1r**, most of the signals of the esters (**1r** and **1s**) are undisturbed; both **1s** and **1r** are different from **1e**, and they are different from each other.

measuring the reaction NMR tubes directly. Although strong proton signals of the excess MTPA chlorides, MTPA acids (hydrolysis products from MTPA chlorides, due to the trace amount of H_2O in deuterated pyridine and the moisture of the experimental environment), and DMAP were present in the ^{1}H NMR spectra, most of the signals of **1r** and **1s** were undisturbed (Fig. 2). It is worthy of note that the ^{1}H NMR chemical shift differences (δ_S–δ_R) of H-24a (+0.013) and H-24b (−0.013) suggested that these two protons should be on different sides of the C-3 MTPA plane. In addition, OH-19 also reacted with (*R*)- and (*S*)-MTPA chloride (due to the presence of DMAP in the reaction tubes) based on the observed ^{1}H NMR chemical shift differences (δ_S–δ_R) of H-12, CH_3-27, CH_3-29 and H-30a (Fig. 3). The absolute stereochemistry of C-3 was determined as *R* based on the observed chemical shift differences (Fig. 3) of **1r** and **1s**. Hence, coussaric acid (**1**) was assigned as (3*R*,4*S*,5*R*,8*R*,9*R*,10*R*,14*S*,17*R*,18*S*,19*S*)-3,19,24-trihydroxy-ursa-12,20(30)-dien-28-oic acid.

A second new 3α,24-hydroxytriterpene acid, 3-*epi*-spathodic acid (**2**), was isolated as its 3,24-diacetate (**2a**). Coussaric acid (**1**) and a mixture of compounds **2**

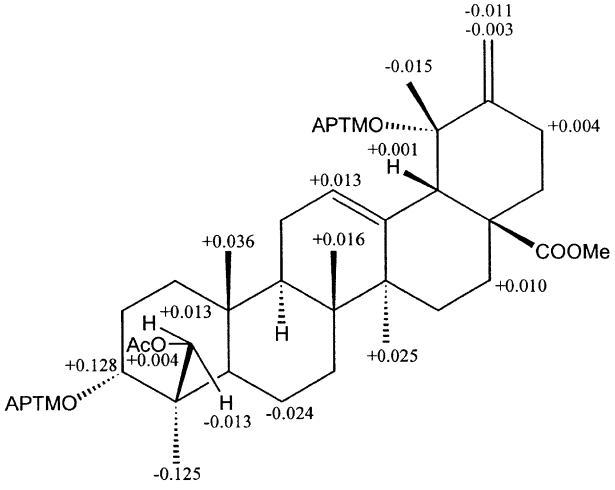

Fig. 3. The values of δ_S–δ_R of MTPA esters (**1s** and **1r**) of **1e**.

and **3** were obtained by HPLC from one major subfraction. The structures of compounds **2** and **3** could be elucidated by careful analysis of the 1D and 2D NMR spectral data of the mixture. However, these two compounds were difficult to separate even using several

different HPLC columns. The ratio of **2** to **3** was determined to be ca. 1:3 based on their ^{1}H NMR spectra. A portion of compound **3** (barbinervic acid) could be obtained in crystalline form from a $CHCl_3$–MeOH (~6:1) solution of the mixture. Then, the remaining mixture was acetylated and separated by HPLC, to give **2a** and **3a**. Both the ^{1}H (Table 1) and ^{13}C (Table 2) NMR spectral data of **2a** were closely comparable to those of **1a**, with the chemical shifts of the protons and carbons of rings A and B of these two compounds being almost identical. The ^{1}H, ^{13}C, DEPT and HMQC spectral data indicated the presence of eight methyl groups in the molecule of **2a**, and two of these methyl groups were assigned to acetyl groups. Thus, **2a** possesses six methyl groups in its skeleton, one methyl more than in **1a**. This is consistent with the absence of any signals observed for an exomethylene in the ^{1}H and ^{13}C NMR spectra of **2a**. Further analysis of the observed HMBC correlations indicated that the gross structure of **2a** was the same as the 3β,24-diacetate of the methyl ester of spathodic acid (DAMESA) (Ngouela et al., 1990). The H-3 signal was observed as a broad singlet at δ_H 5.29 in the ^{1}H NMR spectrum of **2a**, which was very similar to the value obtained for **1a** (δ_H 5.29, *br s*) but clearly different from that reported for DAMESA (δ_H 4.55, *dd*, J = 7.5, 5.0 Hz, H-3α). Furthermore, the chemical shift of C-3 of **2a** (δ_C 73.4) showed a significant upfield shift compared to that of DAMESA (δ_C 80.2). This evidence suggested the OH-3 group to have an α-orientation in **2a**. Very close chemical shifts (partly overlapped) were observed for H-18 and H-19 in the ^{1}H NMR spectrum of **2a** in pyridine-d_5. In order to determine the relative configuration of these two protons, the ^{1}H NMR spectrum of **2a** was run in $CDCl_3$. As expected, the signals of H-18 (δ_H 3.10, *br s*, $W_{1/2}$ ~3.5 Hz) and H-19 (δ_H 3.34, *d*, J = 3.7 Hz) were clearly separated, and the splitting patterns for these two protons were closely comparable to previously reported values for these same protons in analogous triterpenoids (Ngouela et al., 1990; Mimaki et al., 2001; Adnyana et al., 2001). This was further confirmed by the observed correlations from H-18 to H-19 and H-12 in the NOESY spectrum of **2a**. Accordingly, the structure of the second new compound (**2**) was determined as 3-*epi*-spathodic acid.

With the exception of the 3α,24-diacetate of the methyl ester of coussaric acid (**1d**, only 0.1 mg was obtained) and the (*R*)- and (*S*)-MTPA esters of the 24-monoacetate of the methyl ester of coussaric acid (**1r** and **1s**), all isolates and other chemical transformation products obtained in the present study were evaluated for their potential to induce QR (Chang et al., 1997; Misico et al., 2002). However, among the 11 compounds tested, only coussaric acid A (**1**) showed evident induction ability with a CD value of 17.9 μM (8.7 μg/ml), while all the other compounds were considered to be inactive (CD >10 μg/ml). In terms of their potential cancer chemopreventive activity, some naturally occurring and modified triterpenoids have been found to be significant inducers of QR (Ha et al., 2000), inhibitors of Epstein–Barr virus activation and inhibitors of mouse skin carcinogenesis in experimental animals (Konoshima et al., 1996), as well as inhibitors of the production of nitric oxide induced by interferon-γ in mouse macrophages (Honda et al., 2000).

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, and the IR spectrum run on an ATI Mattson Genesis Series FT-IR spectrophotometer. NMR spectral data were recorded at room temperature on a Bruker Avance DPX-300, 360, or DRX-500 MHz spectrometer with TMS as internal standard. Standard pulse sequences were employed for the measurement of 2D NMR spectra (^{1}H–^{1}H COSY, HMQC, HMBC, ROESY, and NOESY). FABMS was obtained on a VG 7070E-HF sector-field mass spectrometer, while HREIMS and EIMS were performed on a Finnigan/MAT 90/95 sector-field mass spectrometer. A YMC-pack ODC-AQ column (5 μm, 25×2 cm i.d., YMC Co., Wilmington, NC) was used for semi-preparative HPLC, along with two Waters 515 HPLC pumps and a Waters 2487 dual λ absorbance detector (Waters, Milford, MA). 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.

X-ray crystallographic analysis data collection for compound **1** was carried out on an Enraf-Nonius Kappa CCD area detector with a rotating anode Mo X-ray tube. The location of non-hydrogen atoms was carried out by direct methods using SIR-92 (Altomare et al., 1993). The WinGX package (Farrugia, 1999) was used for completing the structure determination, and ORTEP (Johnson, 1965; Farrugia, 1997) was used to generate Fig. 1.

3.2. Plant material

The stems (5 kg) of *C. brevicaulis* Krause were collected (collection number 9236) at Estación Experimental ESPOCH (1° 30′ S and 77° 56′ W), Los Vencedores, Canton Puyo, Municipio Puyo, Province Pastaza, Ecuador, in August 1995. A voucher specimen (identified by D.D.S.; accession No. F2156231) is in deposit at the J.D. Searle Herbarium, Field Museum of Natural History, Chicago, IL, USA.

3.3. Quinone reductase induction assay

For the evaluation of plant 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. Plant extracts, chromatographic fractions and pure compounds are considered as inactive when the CD value is >10 μg/ml in the QR assay.

3.4. Extraction and isolation

The dried and milled stems (5 kg) were extracted by maceration with MeOH (3×20 L) at room temperature, for 3 days each. After filtration and evaporation of the solvent under reduced pressure, the combined crude methanolic extract was suspended in H_2O (700 ml) to produce an aqueous MeOH solution (~95%), then partitioned in turn with petroleum ether (3×1000 ml) and EtOAc (3×1000 ml), to afford dried petroleum ether- (12.6 g), EtOAc- (5.8 g), and H_2O-soluble (~75 g) extracts. The concentrations required to double the specific activity of QR for these three extracts were >20, 8.5, and >20 μg/ml, respectively. Therefore, the EtOAc-soluble extract was selected for further detailed purification.

The EtOAc-soluble extract was subjected to chromatography over a Si gel column (5.5×45 cm), eluted with $CHCl_3$–MeOH (50:1, 30:1, 20:1, 15:1, 8:1 and 4:1), to give six fractions (F01–F06). Fraction F02, eluted with $CHCl_3$–MeOH (30:1), was purified over a Si gel column (2.8×35 cm), using *n*-hexanes–EtOAc (2:1) for elution, and afforded stigmasterol (17 mg; 0.00034% w/w) $[\alpha]_D^{23}$ −50.5° (*c* 1.00, $CHCl_3$); lit. $[\alpha]_D^{25}$ −48.5° (*c* 2.00, $CHCl_3$) (Kuksis and Beveridge, 1960] and scutellaric acid (**4**, 15 mg). Fraction F03, eluted with $CHCl_3$–MeOH (20:1), was chromatographed over a Si gel column (2.8×35 cm) using $CHCl_3$–acetone (6:1) as solvent system, to give an additional amount of scutellaric acid (**4**, 10 mg; 0.0005% w/w, total yield) and a subfraction (F0302, 450 mg). Part of this subfraction (150 mg) was then purified by semipreparative HPLC, by eluting with MeOH–H_2O (80:20; 8 ml/min), to afford pure compound **1** ($t_R=11.0$ min, 35 mg; 0.0021% w/w) and a mixture ($t_R=12.7$ min, 86 mg). Barbinervic acid (**3**, 5 mg) was obtained as crystals from a $CHCl_3$–MeOH (~6:1) solution of this mixture. The remaining mixture failed to lead to any additional pure compounds by HPLC using different stationary phases and solvent systems. Thus, it was acetylated using acetic anhydride and pyridine at rt overnight, and the product was then separated by HPLC, eluted with MeOH–H_2O (66:34; 8 ml/min), to afford pure compounds **2a** ($t_R=32.5$ min, 17 mg; 0.001% w/w) and **3a** ($t_R=35.0$ min, 48 mg; 0.0029% w/w). Stigmasterol glucoside (250 mg; 0.005% w/w) $[\alpha]_D^{23}$ −48.2° (*c* 1.00, pyridine); lit. $[\alpha]_D^{26}$ −60.12° (*c* 0.233, pyridine) (Sharma et al., 1997)] was obtained as an amorphous powder from the solution ($CHCl_3$–MeOH, 3:1) of fraction F05, eluted with $CHCl_3$-MeOH (3:1).

3.5. Coussaric acid (*1*)

Colorless needles ($CHCl_3$–MeOH; 5:1), mp 232–233 °C; $[\alpha]_D^{23}$ +51.5° (*c* 1.0, pyridine); UV (MeOH) λ_{max} (log ε) 210 (3.05), 246 (2.53) nm; IR ν_{max} (film) cm^{-1}: 3460, 1690, 1452, 1380, 1201, 899; EIMS *m*/*z* (rel. int.): 486 (4), 468 (18), 450 (10), 440 (25), 424 (22), 189 (33), 244 (28), 223 (24), 219 (32), 217 (46), 216 (58), 206 (86), 201 (42), 187 (58), 175 (100), 159 (37), 145 (39), 132 (63), 105 (44), 43 (44); HREIMS *m*/*z*: 486.3355 $[M]^+$ (calc. for $C_{30}H_{46}O_5$, 486.3345); the ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.6. X-ray crystallography data of coussaric acid (*1*)

A colorless crystal was obtained from $CHCl_3$–MeOH (5:1). The crystal was immersed in Fluorolube oil and cooled to 150 K to minimize crystal degradation and X-ray radiation damages. The data were collected on a Enraf-Nonius Kappa CCD area detector equipped with a rotating anode X-ray generator and MoK_α radiation. Cell parameters: $a=12.0080$ (2) Å; $b=13.6396$ (2) Å; $c=15.6186$ (4) Å; $V=2558.08$ (9) $Å^3$, space group $P2_12_12_1$, $Z=4$, $D_{calc.}=1.264$ g/cm^3, $\lambda=0.71073$ Å, $\mu(MoK_\alpha)=0.084$ mm^{-1}, $F(000)=1064$, $T=150$ K. Data collection yielded 5832 reflections of which all were considered unique. Full-matrix least-squares refinement led to a final *R*, *R* (all), and GOF values of 0.0405, 0.0595, and 0.996. Crystallographic data (excluding structure factors) for the structure of this compound have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 200822. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (Fax: +44-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

3.7. 3,24-Diacetate (*1a*) and 24-monoacetate (*1b*) of coussaric acid

Coussaric acid (**1**) (8.0 mg) was dissolved in 0.3 ml anhydrous pyridine in a 4 ml vial, which was then closed and incubated in a ice-water bath. After 15 min, 0.2 ml acetic anhydride was added to the vial and kept in the ice-water bath for 10 min. The reaction was then quenched by adding 1 ml MeOH to the mixture. After evaporating the solvents under reduced pressure, the

mixture was then purified over a small Si gel column (1.2×15 cm) using $CHCl_3$–MeOH (30:1) for elution, to afford coussaric acid 3,24-diacetate (**1a**, 1.0 mg), coussaric acid 24-monoacetate (**1b**, 4.5 mg) and starting material (**1**, 2.1 mg), in order of polarity. **1a**: $[\alpha]_D^{23}$ +44.6° (*c* 0.53, MeOH); EIMS *m/z* (rel. int.): 570 $[M]^+$ (8), 524 (49), 510 (35), 450 (18), 262 (35), 248 (31), 216 (57), 201 (58), 188 (100), 173 (85), 159 (40), 132 (70), 119 (58), 105 (41), 43 (56); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively. **1b**: $[\alpha]_D^{23}$ +69.4° (*c* 0.38, $CHCl_3$); EIMS *m/z* (rel. int.): 528 $[M]^+$ (9), 510 (34), 482 (27), 262 (25), 216 (17), 173 (100), 145 (23), 132 (54), 119 (62), 105 (45), 43 (58); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.8. Methyl ester of coussaric acid (*1c*)

Coussaric acid (**1**, 7.2 mg) was dissolved in 2 ml $CHCl_3$–MeOH (~4:1), and an excess amount of fresh CH_2N_2 (in Et_2O) was added. The mixture was permitted to stand at rt overnight, to afford the methyl ester of coussaric acid (**1c**). The product was indicated to be highly pure (>99%) by NMR spectral measurements without any purification. **1c**: $[\alpha]_D^{23}$ +80.0° (*c* 0.15, MeOH); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.9. 3α,24-Diacetate of the methyl ester of coussaric acid (*1d*) and 24-monoacetate of the methyl ester of coussaric acid (*1e*)

Compound **1c** (4.5 mg) was selectively acetylated using the method described above for obtaining the diacetate **1a** and the monoacetate **1b** from coussaric acid (**1**). The products were separated on a Si gel column (1.2×15 cm) by elution with *n*-hexanes–EtOAc (5:2), to afford the 3α,24-diacetate of the methyl ester of coussaric acid (**1d**, 0.1 mg) and the 24-monoacetate of the methyl ester of coussaric acid (**1e**, 2.2 mg). Elution of the column with *n*-hexanes–EtOAc–MeOH (20:20:1) gave the starting material, the methyl ester of coussaric acid (**1c**, 0.7 mg). ^1H NMR data of **1d** (500 MHz, $CDCl_3$, TMS): δ 5.45 (1H, *t*, H-12), 5.02 (1H, *br s*, OH-19), 4.95 (1H, *br s*, H-30a), 4.79 (1H, *br s*, H-30b), 4.69, (1H, *br s*, H-3), 4.19 (1H, *d*, *J*=11.7 Hz, H-24a), 3.97 (1H, *d*, *J*=11.7 Hz, H-24b), 3.62 (3H, *s*, COOMe), 2.91 (1H, *s*, H-18), 2.09 (3H, *s*, OAc), 2.05 (3H, *s*, OAc). **1e**: $[\alpha]_D^{23}$ +65.2° (*c* 0.20, $CHCl_3$); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.10. Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of *1e* by a convenient Mosher ester procedure

Two portions of compound **1e** (each 0.6 mg) were separately treated with (*S*)-(+)- and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (3 μl) in deuterated pyridine (0.5 ml) directly in NMR tubes as described previously (Su et al., 2002), and afforded the (*R*)- and (*S*)-MTPA ester, respectively. It was necessary to add a small amount (~0.2 mg) of the catalyst *N,N*-dimethyl-4-aminopyridine to both tubes to accelerate the reactions. ^1H NMR data of the (*R*)-MTPA ester derivative (**1r**) of **1e**: (500 MHz, pyridine-d_5; data were obtained from the reaction NMR tube directly and were assigned based on the correlations of the ^1H–^1H COSY and NOESY spectra) δ 5.491 (1H, *br s*, H-12), 5.423 (1H, *br s*, H-3), 4.980 (1H, *s*, H-30a), 4.799 (1H, *s*, H-30b), 4.470 (1H, *d*, *J*=11.5 Hz, H-24a), 4.146 (1H, *d*, *J*=11.5 Hz, H-24b), 3.201 (1H, *td*, *J*=13.5, 4.2 Hz, H-16a), 3.095 (1H, *td*, *J*=13.1, 4.2 Hz, H-21a), 2.997 (1H, *br s*, H-18), 2.051 (3H, *s*, OAc), 1.661 (3H, *s*, CH_3-27), 1.612 (3H, *s*, CH_3-29), 1.517 (1H, *m*, H-6a), 1.205 (3H, *s*, CH_3-23), 0.877 (3H, *s*, CH_3-25), 0.823 (3H, *s*, CH_3-26); ^1H NMR data of the (S)-MTPA ester derivative (**1s**) of **1e**: (500 MHz, pyridine-d_5; data were obtained from the reaction NMR tube directly and were assigned based on the correlations of the ^1H–^1H COSY and NOESY spectra) δ 5.550 (1H, *br s*, H-3), 5.505 (1H, *br s*, H-12), 4.969 (1H, *s*, H-30a), 4.797 (1H, *s*, H-30b), 4.483 (1H, *d*, *J*=11.4 Hz, H-24a), 4.133 (1H, *d*, J=11.4 Hz, H-24b), 3.200 (1H, *td*, J=13.4, 4.2 Hz, H-16a), 3.100 (1H, *td*, *J*=13.2, 4.2 Hz, H-21a), 2.998 (1H, *br s*, H-18), 2.055 (3H, *s*, OAc), 1.685 (3H, *s*, CH_3-27), 1.597 (3H, *s*, CH_3-29), 1.493 (1H, *m*, H-6a), 1.080 (3H, *s*, CH_3-23), 0.912 (3H, *s*, CH_3-25), 0.841 (3H, *s*, CH_3-26).

3.11. Barbinervic acid (*3*)

Colorless needles ($CHCl_3$–MeOH, 6:1), mp 278–280 °C; $[\alpha]_D^{23}$ +32.5° (*c* 0.25, pyridine); $[\alpha]_D^{23}$ +21.8° (*c* 0.12, $CHCl_3$) [lit. $[\alpha]_D^{20}$ +12° (*c* 0.40, $CHCl_3$) (Delle Monache et al., 1996)]; EIMS *m/z* (rel. int.): 488 $[M]^+$ (3), 470 (7), 442 (26), 370 (12), 264 (47), 246 (74), 231 (38), 219 (42), 206 (83), 187 (39), 175 (90), 146 (100), 119 (57); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.12. 3,24-Diacetate 3-epi-spathodic acid (*2a*) and 3,24-diacetate barbinervic acid (*3a*)

A mixture (24 mg) of 3-*epi*-spathodic acid (**2**) and barbinervic acid (**3**) was treated with pyridine (0.5 ml) and acetic anhydride (0.5 ml) at rt overnight. The products were separated by HPLC, eluted with MeOH–H_2O (66:14) at 8 ml/min, to afford 3-*epi*-spathodic acid 3,24-diacetate (**2a**, 7.5 mg, t_R=31.2 min) and barbinervic acid 3,24-diacetate (**3a**, 16.0 mg, t_R=32.5 min). **2a**: $[\alpha]_D^{23}$ −0.6° (*c* 1.0, MeOH); EIMS *m/z* (rel. int.): 572 $[M]^+$ (1), 554 (1), 512 (7), 264 (75), 246 (92), 231 (49), 201 (100), 188 (34), 131 (23), 119 (24), 105 (17), 43 (20); HRFABMS *m/z*: 595.3624 $[M+Na]^+$ (calc. for $C_{34}H_{52}O_7Na$, 595.3611); ^1H NMR data in

$CDCl_3$ (300 MHz, TMS): δ 5.45 (1H, *br s*, H-12), 4.98 (1H, *br s*, H-3), 4.19 (1H, *d*, $J=11.3$ Hz, H-24a), 3.93 (1H, *d*, $J=11.3$ Hz, H-24b), 3.34 (1H, *d*, $J=3.7$ Hz, H-19), 3.10 (1H, *br s*, $W_{1/2}$ ~3.5 Hz, H-18), 2.10 (3H, *s*, OAc-3), 2.03 (3H, *s*, OAc-24), 1.30 (3H, *s*, CH_3), 0.99 (3H, *s*, CH_3), 0.94 (6H, *s*, $2\times CH_3$), 0.69 (3H, *s*, CH_3); for ^1H and ^{13}C NMR data in pyridine-d_5, see Tables 1 and 2, respectively. **3a**: $[\alpha]_D^{23}$ −8.2° (*c* 1.2, MeOH); EIMS *m/z* (rel. int.): 572 $[M]^+$ (2), 526 (46), 512 (21), 454 (15), 264 (12), 246 (31), 218 (26), 201 (44), 188 (78), 173 (37), 146 (100), 119 (36), 43 (31); for ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively.

3.13. Scutellaric acid (4)

White amorphous powder, mp 273–276 °C; $[\alpha]_D^{23}$ +34.6° (*c* 0.85, pyridine) [lit. $[\alpha]_D^{25}$ +31° (*c* 0.170, MeOH) (Deepak and Handa, 1998)]; ^1H and ^{13}C NMR (Morota et al., 1995) spectral data consistent with literature values.

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