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Secondary metabolites from *Commiphora opobalsamum* and their antiproliferative effect on human prostate cancer cells

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Abstract

A cycloartane-type triterpenoid (**1**), an aliphatic alcohol glycoside (**2**), an eudesmane-type sesquiterpenoid (**3**), and a guaiane-type sesquiterpenoid (**4**) were isolated from the resinous exudates of *Commiphora opobalsamum* along with six known sesquiterpenoids (**5**–**10**). Their structures were established by extensive analysis of their 1D and 2D NMR spectroscopic data and chemical methods. The isolated compounds **1**–**3** and **5**–**9** were tested against human prostate cancer cell PC 3 and LNCaP. Among them, **1** and **2** showed moderate antiproliferative effects on human prostate cancer cell lines with IC_{50} values ranging from 5.7 to 23.6 μM; they were also able to inhibit the expression of androgen receptor (AR) in LNCaP cells. The six sesquiterpenoids were inactive in the bioassays.

Keywords: *Commiphora opobalsamum*; Burseraceae; Triterpenoid; Aliphatic alcohol glycoside; Sesquiterpenoid; Antiproliferative; Prostate cancer cells

1. Introduction

The genus *Commiphora* of the Burseraceae family includes over 150 species and is mainly distributed in Eastern Africa, Arabia and India (Vollesen, 1989). The resinous exudates of these plant species were found to be of significant biological value for their cytotoxic, anti-inflammatory, and antimicrobial effects, and they have been used in dentistry for endodontic therapy and temporary fillings, as well as in antiseptics, soaps and deodorants (Dolara et al., 2000; Habtemariam, 2003; Francisa et al., 2004; Meeker and Linke, 1988; Shapiro et al., 1994; Soderberg et al., 1996).

Previous phytochemical investigations on this genus mainly focused on the species *Commiphora myrrha* (Zhu et al., 2001b, 2003), *C. mukul* (Patil et al., 1972, 1973a,b), *C. wightii* (Fatope et al., 2003; Zhu et al., 2001a), *C. molmol* (Brieskorn and Noble, 1980, 1983a,b), *C. pyracanthoides* (Thomas, 1961; Thomas et al., 1961; Thomas and Willhalm, 1964) *C. erlangeriana* (Dekebo et al., 2002a; Habtemariam, 2003), and *C. sphaerocarpa* (Dekebo et al., 2000, 2002b), leading to isolation of a series of terpenoids, steroids, flavonoids and carbohydrates (Ashry et al., 2003; Hanuš et al., 2005). However, the species *C. opobalsamum* has hitherto not been chemically studied. In the course of our search for potential antiproliferative natural products using primary human prostate cancer cell lines (Yuan et al., 2004), the petroleum ether extract of the resinous exudates of *C. opobalsamum* exhibited moderate inhibitory activity against PC3 human prostate cancer cells with an IC_{50} of 31 μM. Chemical investigation of the active fraction led to the isolation of four new compounds (**1**–**4**) together with six known sesquiterpenoids (**5**–**10**). In addition, the antiproliferative effect of the isolated compounds against human prostate cancer cell lines was also evaluated.

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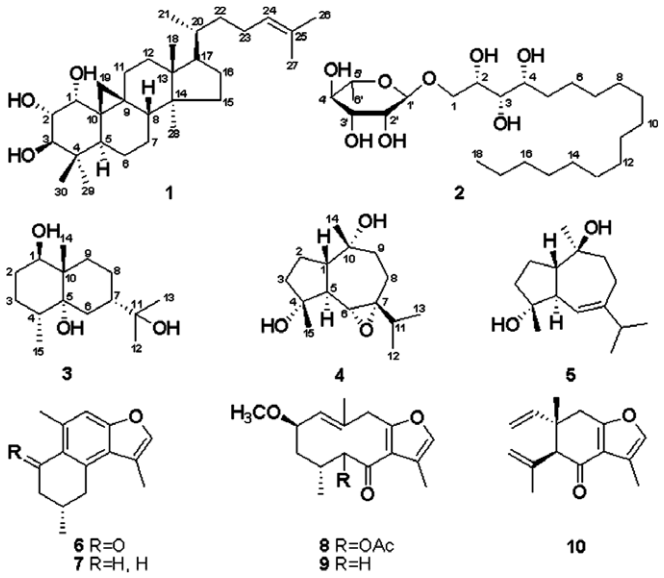

2. Results and discussion

2.1. Isolation and structural elucidation of new compounds

The petroleum ether extract of the resinous exudates of *C. opobalsamum* was subjected to column chromatography on silica gel and Sephadex LH-20. Repeated chromatography led to the isolation of a new triterpenoid, cycloartane-24-en-1α,2α,3β-triol (**1**), an aliphatic alcohol glycoside, octadecane-1,2*S*,3*S*,4*R*-tetrol 1-*O*-α-L-rhamnopyranoside (**2**), and two new sesquiterpenoids eudesmane-1β,5α,11-triol (**3**) and guaia-6α,7α-epoxy-4α,10α-diol (**4**), along with six known compounds (**5**–**10**).

Compound **1** was isolated as colorless flakes. The molecular formula, $C_{30}H_{50}O_3$, was established by HREIMS at *m/z* 458.3781 $[M]^+$ (Calc. for $C_{30}H_{50}O_3$, 458.3760). A total of 30 carbons were resolved in the ^{13}C NMR spectrum (Table 1). The ^1H NMR spectrum showed the presence of six tertiary methyls, a secondary methyl (δ_H 0.90, *d*, $J = 6.4$ Hz), an olefinic proton (δ_H 5.11, *t*, $J = 7.0$ Hz), and three methine protons geminal to hydroxyl groups (δ_H 3.50, 3.56, and 3.65), as analyzed with the aid of 2D NMR spectroscopic data. In addition, a pair of cyclopropane methylene protons (δ_H 0.51 and 0.73, each *d*, $J = 4.3$ Hz) was observed, which supported the presence of the cycloartane-type skeleton. ^1H–^1H COSY coupling between δ_H 3.50 (H-3), 3.65 (H-2) and 3.56 (H-1), together with HMBC correlations from δ_H 3.50 (H-3) to the carbon resonances at δ_C 35.9 (C-4), 25.6 (C-29), 14.2 (C-30) and 72.5 (C-2) established the assignments of the oxymethine protons to be at H-1, H-2, and H-3, respectively. The relative stereochemistry was established by analysis of NOESY correlations (Fig. 1). Correlations of H-19a to H-1 and H-2; H-2 to H-30; and H-3 to H-29 and H-5 suggested that three hydroxyl groups at C-1, 2 and 3 were α, α, and β oriented, respectively. Based on the comparison of its NMR spectroscopic data with those of cycloartenol (Seo et al., 1988; Nes et al., 1998), the structure of **1** was assigned as cycloartane-24-en-1α,2α,3β-triol, and consistent with ^1H–^1H COSY, HMQC and HMBC correlations.

Table 1
^{13}C and ^1H NMR spectroscopic data and HMBC of compound **1** in $CDCl_3$

No.	δ_C[a]	δ_H[b]	HMBC (H to C)
1	75.3	3.56 (1H, *br s*)	C-2, 3, 5, 10
2	72.5	3.65 (1H, *br d*, $J = 7.9$)	C-3
3	78.1	3.50 (1H, *d*, $J = 9.8$)	C-2, 4, 29, 30
4	40.1		
5	39.3	1.94 (1H, *dd*, $J = 4.3, 12.8$)	C-3, 4, 6, 10, 19, 29, 30
6α	20.6	0.85 (1H, *m*)	C-5, 7
β		1.65 (1H, *m*)	C-5
7α	25.6	1.16 (1H, *m*)	C-5, 6, 8
β		1.36 (1H, *m*)	C-5, 6, 8
8	47.9	1.53 (1H, *dd*, $J = 4.5, 10.2$)	C-6, 7, 9, 10, 11, 14, 15, 19, 28
9	20.3		
10	29.0		
11α	26.1	2.29 (1H, *m*)	C-9, 12, 19
β		1.27 (1H, *m*)	C-9, 12, 13, 19
12	32.7	1.68 (2H, *m*)	C-11, 13, 14, 18
13	48.1		
14	48.8		
15	35.7	1.32 (2H, *m*)	C-8, 14, 16, 17, 21, 28
16α	28.1	1.91 (1H, *m*)	C-14, 15, 17
β		1.32 (1H, *m*)	C-14, 15, 17
17	52.2	1.60 (1H, *m*)	C-13, 14, 16, 18, 21, 20, 22
18	18.1	0.97 (3H, *s*)	C-12, 13, 14, 17
19a	29.4	0.51 (1H, *d*, $J = 4.3$)	C-1, 5, 8, 9, 10, 11
19b		0.73 (1H, *d*, $J = 4.3$)	C-1, 5, 8, 9, 10, 11
20	35.9	1.32 (1H, *m*)	C-17, 21, 23
21	18.2	0.90 (3H, *d*, $J = 6.4$)	C-17, 20, 22
22	36.3	1.05 (1H, *m*)	
		1.42 (1H, *m*)	C-20, 21, 23
23	24.9	1.88 (1H, *m*)	C-20, 22, 24, 25
		2.06 (1H, *m*)	C-22, 24, 25
24	125.2	5.11 (1H, *t*, $J = 7.0$)	C-22, 23, 26, 27
25	131.0		
26	17.7	1.62 (3H, *s*)	C-24, 25, 27
27	25.7	1.69 (3H, *s*)	C-24, 25, 26
28	19.4	0.97 (3H, *s*)	C-8, 13, 14, 15
29	25.6	1.01 (3H, *s*)	C-3, 4, 5, 30
30	14.2	0.83 (3H, *s*)	C-3, 4, 5, 29

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz. Data assignments were based on ^1H–^1H COSY, HMQC, and HMBC experiments and comparison with the literature.

Compound **2** was obtained as white feathery crystals, and its molecular formula $C_{24}H_{48}O_8$ was determined by analysis of positive HRESIMS at *m/z* 487.3238 $[M + Na]^+$ (Calc. for $C_{24}H_{48}O_8Na$, 487.3247). The ^{13}C and ^1H NMR spectra showed the presence of a sugar and a long-chain aliphatic moiety (Table 2). The aglycone of octadecane-1,2,3,4-tetrol was deduced from analysis of the HMQC and ^1H–^1H COSY; its aglycone has been previously isolated from *C. mukul* (Patil et al., 1973b). The ^1H NMR spectrum showed an anomeric proton signal at δ_H 4.51 (*br s*) and a methyl resonance at δ_H 1.11 (3H, *d*, $J = 6.2$ Hz). In addition, the car-

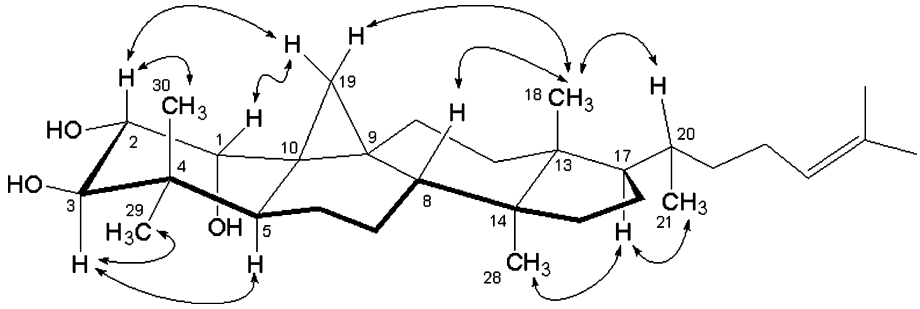

Fig. 1. Key NOESY (↔) correlations of 1.

Table 2
^{13}C and ^{1}H NMR spectroscopic data of compound **2** in DMSO

No.	δ_C[a]	δ_H[b]
1	70.7	3.38 (2H, *m*)
2	68.6	3.47 (1H, *m*)
3	70.3	3.61 (1H, *m*)
4	70.9	3.43 (1H, *m*)
5	25.7	1.33 (2H, *m*)
6	33.4	1.28 (2H, *m*)
7	29.5	1.23 (2H, *br s*)
8–14	29.3(7C)	1.23 (14H, *br s*)
15	29.0	1.23 (2H, *br s*)
16	31.5	1.23 (2H, *br s*)
17	22.3	1.23 (2H, *br s*)
18	14.2	0.84 (3H, *t*, $J = 6.7$)
1′	100.6	4.51 (1H, *br s*)
2′	71.2	3.57 (1H, *br s*)
3′	73.1	3.18 (1H, *m*)
4′	72.2	3.16 (1H, *m*)
5′	68.8	3.38 (1H, *m*)
6′	18.2	1.11 (3H, *d*, $J = 6.2$)
2-OH		4.22 (1H, *d*, $J = 5.6$)
3-OH		4.50 (1H, *d*, $J = 5.7$)
4-OH		4.52 (1H, *d*, $J = 6.0$)
2′-OH		4.70 (1H, *d*, $J = 4.3$)
3′-OH		4.21 (1H, *d*, $J = 6.1$)
4′-OH		4.72 (1H, *d*, $J = 5.4$)

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz. Data assignments were based on ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

Table 3
^{13}C and ^{1}H NMR spectroscopic data of compounds **3** and **4** in $CDCl_3$

No.	3		4	
	δ_C[a]	δ_H[b]	δ_C [a]	δ_H[b]
1	76.8	3.76 (1H, *br d*, $J = 11.6$)	50.5	1.54 (1H, *m*)
2α	25.7	1.52 (1H, *m*)	23.8	1.33 (1H, *m*)
β		1.60 (1H, *m*)		1.54 (1H, *m*)
3α	27.3	2.03 (1H, *m*)	40.0	1.84 (1H, *m*)
β		1.37 (1H, *m*)		1.73 (1H, *m*)
4	39.7	1.75 (1H, *m*)	79.3	
5	89.1		54.5	1.60 (1H, *dd*, $J = 10.0, 12.6$)
6α	37.1	1.86 (1H, *m*)	72.1	3.81 (1H, *d*, $J = 10.0$)
β		1.94 (1H, *m*)		
7	43.9	1.89 (1H, *br s*)	86.4	
8α	24.7	1.75 (1H, *m*)	29.1	1.94 (1H,*m*)
β		1.60 (1H, *m*)		1.73 (1H, *m*)
9α	34.6	1.84 (1H, *m*)	31.8	1.84 (1H, *m*)
β		1.37 (1H, *m*)		1.46 (1H, *dt*, $J = 10.5, 5.1$)
10	43.7		83.1	
11	81.1		32.5	1.94 (1H, *m*)
12	22.6	1.33 (3H, *s*)	17.1	1.04 (3H, *d*, $J = 6.8$)
13	30.2	1.15 (3H, *s*)	18.6	1.05 (3H, *d*, $J = 7.0$)
14	15.5	1.03 (3H, *s*)	23.1	1.25 (3H, *s*)
15	17.6	1.01 (3H, *d*, $J = 7.8$)	25.2	1.34 (3H, *s*)

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz. Data assignments were based on ^{1}H–^{1}H COSY, HMQC, and HMBC experiments.

bon signals at δ_C 100.6 (C-1′), 73.1 (C-3′), 72.2 (C-4′), 71.2 (C-2′), 68.8 (C-5′) and 18.2 (C-6′) were observed in the ^{13}C NMR spectrum. This suggested the presence of L-rhamnose with an α-configuration from the very low *J* value of its anomeric proton (Flamini et al., 2000). In the HMBC spectrum, a correlation between the anomeric proton (δ_H 4.51) and C-1 (δ_C 70.7) determined the α-L-rhamnosyl unit to be linked to the C-1 position. Acid hydrolysis of **2** gave the aglycone of octadecane-1,2*S*,3*S*,4*R*-tetrol (**2a**) and rhamnose. The sugar unit was identified by both TLC and comparison with authentic samples. The stereochemistry of compound **2a** was deduced as 2*S*, 3*S*, and 4*R*, by comparison of both the melting point and optical rotation data reported for octadecane-1,2,3,4-tetrol isomers (Kumar and Dev, 1987). The data measurements for **2a** (m.p. 78–81 °C and $[\alpha]_D^{20}$+15.1) were consistent with that for naturally occurring guggultetrol-18 (2*S*, 3*S*, 4*R*). Therefore, the structure of compound **2** was identified to be octadecane-1,2*S*,3*S*,4*R*-tetrol 1-*O*-α-L-rhamnopyranoside.

Compound **3**, obtained as colorless crystals, exhibited a molecular ion peak $[M]^+$ at *m*/*z* 256.6 in the ESIMS, consistent with the molecular formula $C_{15}H_{28}O_3$ deduced from HREIMS (*m*/*z* 238.1936 $[M - H_2O]^+$; Calc. for $C_{15}H_{26}O_2$, 238.1933). The ^{1}H NMR spectrum showed the presence of three methyl singlets (δ_H 1.03, 1.15, and 1.33), one methyl doublet (δ_H 1.01, *d*, $J = 7.8$ Hz) and one oxymethine proton (δ_H 3.76, *br d*, $J = 11.6$ Hz). The ^{13}C NMR spectrum showed 15 carbon signals (Table 3), which were determined with the aid of analysis of its HMQC spectrum to be four methyls, five methylenes, three methines (one oxygenated), and three quaternary carbons (two oxygenated), suggesting that compound **3** was a sesquiterpenoid. The structural determination was provided by analysis of the 2D NMR spectroscopic data, including ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. The ^{1}H–^{1}H COSY led to the

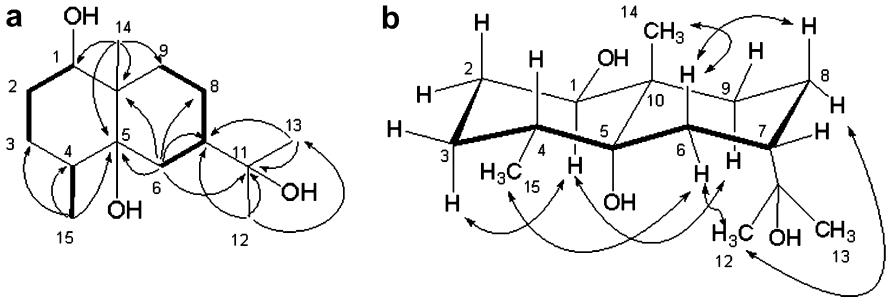

Fig. 2. (a) Key ^{1}H–^{1}H COSY (—) and HMBC (H → C) correlations of 3; (b) Key NOESY (↔) correlations of 3.

establishment of three partial structures as depicted in bold lines (Fig. 2a). Connection of these fragments to give the eudesmane skeleton was furnished by HMBC correlations of δ_H 1.03 (H-14) to δ_C 76.8 (C-1), 89.1 (C-5), 34.6 (C-9), and 43.7 (C-10); δ_H 1.01 (H-15) to δ_C 27.3 (C-3), 39.7 (C-4), and 89.1 (C-5); δ_H 1.86 (H-6α) to δ_C 89.1 (C-5) and 43.7 (C-10); and δ_H 1.33 (H-12) and 1.15 (H-13) to δ_C 43.9 (C-7) and 81.1 (C-11) (Fig. 2a). The above NMR spectroscopic data showed that **3** had the same partial structure as canusesnol C previously isolated from *Capsicum annuum* (Kawaguchi et al., 2004). The NOESY correlations of H-6β to H-8β and H-14; and H-1α to H-9α and H-3α (Fig. 2b) indicated that all these substituents must be axial, and a *transoid* relationship of the ring junction was established. Additional NOESY correlations of H-6α to H-12 and H-15; and H-8α to H-12 finalized the configurations of 1-OH, 5-OH, the C-7 isopropyl group, Me-14, and Me-15 to be β-, α-, α-, β- and α-oriented, respectively. Thus, the structure of compound **3** was assigned as eudesmane-1β,5α,11-triol.

Compound **4** was isolated as a yellow oil, and its molecular formula was deduced as $C_{15}H_{26}O_3$ from its HREIMS at *m/z* 254.1882 (Calc. for $C_{15}H_{26}O_3$, 254.1882), which was an oxygen atom more than the known compound guaianediol (**5**) (Sayed and Hamann, 1996). The ^{1}H NMR spectrum showed the presence of two methyl singlets (δ_H 1.25 and 1.34), two methyl doublets (δ_H 1.04, *d*, $J = 6.8$ Hz and δ_H 1.05, *d*, $J = 7.0$ Hz), one oxymethine proton (δ_H 3.81, *d*, $J = 10.0$ Hz). The ^{13}C NMR spectrum (Table 3) exhibited signals for 15 carbons including four methyls, four methylenes, four methines (one oxygenated), and three oxygenated quaternary carbons. The above data suggested that the structure of **4** was very similar to that of **5**, which was verified by analysis of its 2D NMR spectra, including HMQC,^{1}H–^{1}H COSY and HMBC (Fig. 3a). The only difference was the observation of a doublet at δ_H 3.81 ($J = 10.0$ Hz) instead of the olefinic proton signal for **5** at δ_H 5.49. Correspondingly, the C-6 and C-7 carbon resonances shifted from δ_C 121.3 and 149.6 to δ_C 72.1 and 86.4, respectively. Based on the above analysis, a 6,7-epoxy group was established for **4**. The relative stereochemistry was established from a NOESY experiment. In this spectrum, no NOESY correlation was observed between the two bridgehead protons, which indicated a *transoid* relationship for H-1 and H-5. While the NOESY spectrum of **4** showed correlations of H-1 to H-14 and H-15; H-6 to H-12 and H-15; H-5 to H-8α; as well as H-8β to H-12 (Fig. 3b). Therefore, the structure of compound **4** was established as guaia-6α,7α-epoxy-4α,10α-diol.

The structures of the known isolated compounds (**5**–**10**) from *C. opobalsamum* were determined as guaianediol (**5**) (Sayed and Hamann, 1996), myrrhone (**6**) (Zhu et al., 2003), dihydropyrocurzerenone (**7**) (Dekebo et al., 2002b), 2-methoxy-5-acetoxy-furanogermacr-1(10)-en-6-one (**8**) (Brieskorn and Noble, 1980), (1(10)*E*,2*R*,4*R*)-2-methoxy-8,12-epoxygermacra-1(10),7,11-trien-6-one (**9**) (Dekebo et al., 2000), and curzerenone (**10**) (Dekebo et al., 2002b) by comparison of their ^{1}H and ^{13}C NMR, as well as MS data with those reported in the literature.

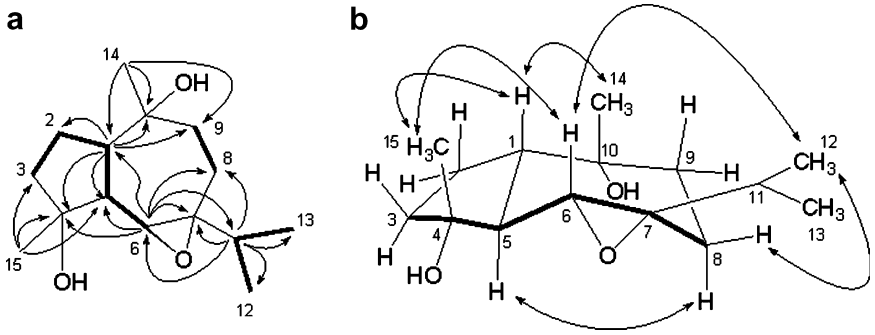

Fig. 3. (a) Key ^{1}H–^{1}H COSY (—) and HMBC (H → C) correlations of **4**; (b) Key NOESY (↔) correlations of **4**.

2.2. Antiproliferative effect on human prostate cancer cells

The antiproliferative effects of compounds **1**–**3** and **5**–**9** were evaluated against PC 3 and LNCaP human prostate cancer cells. Among them, **1** and **2** exhibited moderate antiproliferative effects against PC 3 cells with the IC_{50} values of 5.7 and 7.1 μM, respectively. However, **1** and **2** were less active against LNCaP cells with IC_{50} values of 22.1 and 23.6 μM, respectively. No antiproliferative effects for compounds **3** and **5**–**9** were observed.

Androgens play an important role in the proliferation, differentiation, maintenance, and function of the prostate, and are also involved in the development and progression of prostate cancer (Hovenian and Deming, 1948). The androgen receptor (AR), a transcription factor that binds with androgens to specific DNA sites, is still expressed and functional in many advanced or hormone refractory prostate cancers. Minimization or elimination of the function of AR has been suggested to be potentially an effective treatment for repressing the development and progression of the cancer (Grossmann et al., 2001).

To determine whether AR protein levels are changed with the treatment of compounds **1** and **2**, western blot analysis was performed in an androgen-responsive LNCaP prostate cancer cell line. Fig. 4 showed that AR protein levels were decreased with **1** and **2** at 10 μM in the presence or absence of mibolerone (Mib). Compared with **2**, compound **1** possessed a more significant inhibitory effect on the expression of AR in LNCaP cells.

In order to ascertain if compounds **1** and **2** are actually able to block androgen action, transient transfection was performed to measure the androgen-dependent secretion of prostate-specific antigen (PSA). The results in Fig. 5 showed that the PSA protein level decreased with the treatment of compounds **1** and **2** at a concentration of 10 μM, suggesting that androgen action was inhibited. Although **1** was more efficient in inhibiting AR expression, it was less effective than **2** in the transient transfection assay.

Since compounds **1** and **2** exhibited antiproliferative effects, the antiproliferative mechanism of these principles against human prostate cancer cell lines is being investigated in more detail.

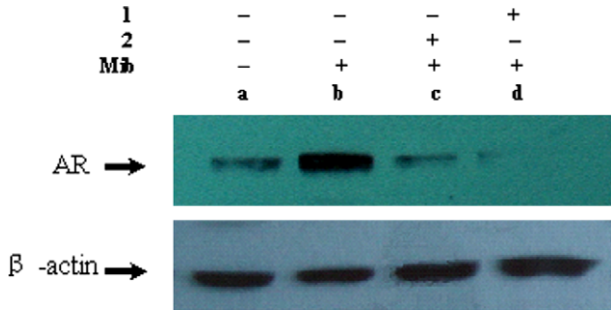

Fig. 4. Western blot analysis of AR expression level in LNCaP cells. The whole cell lysates from LNCaP cells treated with or without the above chemical was analyzed by western blot. β-actin served as an internal control to monitor protein loading and transfer efficiency.

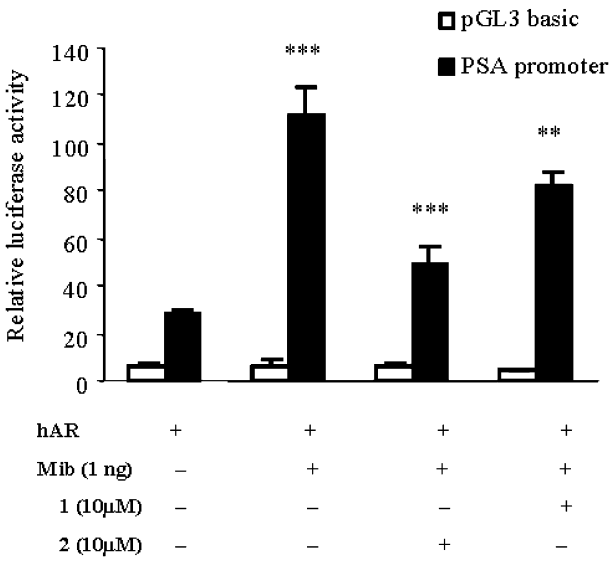

Fig. 5. Inhibitory effects of 1 and 2 on AR transcription activity. LNCaP cells were cotransfected with human expression vector hAR and pGL3-PSA6kb promoter luciferase reporter. The pGL3 basic vector was included as a control. Cells were incubated with 1 nmol/L Mib or 1 nmol/L Mib plus indicated amount of 1 or 2 for 24 h after transfection. $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control.

3. Experimental

3.1. General methods

Melting points were determined on an X-6 melting-point apparatus (Beijing TECH Instrument Co. Ltd.) and were uncorrected. Optical rotations were measured on a Perkin–Elmer 241 MC polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 600 spectrometer at 600 (^{1}H) and 150 (^{13}C) MHz. EIMS and HREIMS spectra were recorded on a Finnigan MAT-95 mass spectrometer and a Waters GCT system, respectively. ESIMS and HRESIMS spectra were measured on an API 4000 mass spectrometer and a Finnigan LC Q^{DECA} instrument. Column chromatography (CC) was performed on silica gel (200–300 mesh) or Sephadex LH-20 (Amersham Biosciences). TLC was carried out with glass pre-coated silica gel GF_{254} plates (Qingdao Haiyang Chemical Co. Ltd.). Spots were visualized under iodine vapor or by spraying with 10% H_2SO_4–EtOH followed by heating.

3.2. Plant material

The exudates of *C. opobalsamum* were purchased in September 2002 from the Affiliated Hospital of Shandong Traditional Chinese Medical University, Jinan, PR China. It was imported from India and identified by Prof. Qishi Sun, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, PR China. A voucher specimen (No. 20020910CO) has been deposited at the Laboratory of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University, PR China.

3.3. Extraction and isolation

The exudates of *C. opobalsamum* (3 kg) were pulverized and extracted with petroleum ether (PE) for 36 h. The extract (270 g) was then subjected to CC over silica gel and eluted with a PE–EtOAc gradient of increasing amount of EtOAc to provide eight fractions (A–H). Fraction A was further purified by preparative TLC (PE) to afford compound **10** (70 mg). Fraction C was subjected to silica gel CC and eluted with PE–EtOAc (100:1) to yield compounds **6** (81 mg) and **7** (25 mg), which were further purified by preparative TLC and recrystallization, respectively. Fraction D was then purified using silica gel CC eluted with a PE–EtOAc gradient to afford compound **8** (290 mg) and eight subfractions (D1–D8). Compound **9** (59 mg) was isolated from subfraction D1 eluted with PE–EtOAc (95:5). Further purification of subfraction D3 by silica gel CC eluted with PE–EtOAc (9:1) and recrystallization in $CHCl_3$ afforded compound **3** (4.2 mg). Fraction F eluted with PE–Me_2CO (93:7) gave five subfractions (F1–F5). Subfraction F3 was subjected to silica gel CC eluted with PE–Me_2CO (9:1) and Sephadex LH20 CC with $CHCl_3$–MeOH (1:1) to give compound **4** (6 mg). Compound **5** (6 mg) was obtained from subfraction F5 by silica gel CC eluted with *n*-hexane-$CHCl_3$–MeOH (100:100:3). Fraction G was subjected to silica gel CC and eluted with a gradient of PE-Me_2CO to give compound **2** (52 mg) and four subfractions (G1–G4). Compound **1** (120 mg) was obtained from subfraction G3 by silica gel CC eluted with PE–Me_2CO (85:15) and further purified by recrystallization.

3.4. Cycloartane-24-en-1α,2α,3β-triol (*1*)

Colorless flakes; m.p. 144–146 °C; $[\alpha]_D^{20}$ +113.4 ($CHCl_3$; c 1.78); for ^1H and ^{13}C NMR ($CDCl_3$), see Table 1; HREIMS *m/z* 458.3781 $[M]^+$ (Calc. for $C_{30}H_{50}O_3$, 458.3760); ESIMS *m/z* 481.6 $[M + Na]^+$.

3.5. Octadecane-1,2S,3S,4R-tetrol 1-O-α-L-rhamnopyranoside (*2*)

White feathery crystals; m.p. 126–129°C; $[\alpha]_D^{20}$ −22.2 (EtOH; c 0.9); for ^1H and ^{13}C NMR (DMSO-d_6) see Table 2; HRESIMS *m/z* 487.3238 $[M + Na]^+$ (Calc. for $C_{24}H_{48}O_8Na$, 487.3247); ESIMS *m/z* (rel. int.): 487.2 $[M + Na]^+$ (100), 465.7 $[M + H]^+$ (10).

3.6. Acid hydrolysis of *2*

Compound **2** (15 mg) was dissolved in a mixture of 10% HCl (2 ml) and MeOH (2 ml), and refluxed at 50 °C for 12 h. Then, H_2O (20 ml) was added and extracted with EtOAc. The solvent was removed, and the residue was subjected to CC over silica gel and eluted with $CHCl_3$–MeOH (15:1) to give **2a**(8 mg). The sugar was identified by TLC and compared with authentic samples.

3.7. Octadecane-1,2S,3S,4R-tetrol (*2a*)

White powders; m.p. 78–81 °C; $[\alpha]_D^{20}$ +15.1 ($CHCl_3$; c 1.85); ^{13}C NMR ($CDCl_3$) δ: 73.4 (C-3), 73.2 (C-2), 72.8 (C-4), 64.5 (C-1), 33.4 (C-5), 31.6 (C-16), 29.4-29.5 (9C, C-7 to C-15), 25.6 (C-6), 22.7 (C-17), 13.8 (C-18).

3.8. Eudesmane-1β,5α,11-triol (*3*)

Colorless crystals; m.p. 96–98°C; $[\alpha]_D^{20}$ −33.4 ($CHCl_3$; c 0.84); ^1H and ^{13}C NMR ($CDCl_3$) see Table 3; HREIMS *m/z* 238.1936 $[M - H_2O]^+$ (Calc. for $C_{15}H_{26}O_2$, 238.1933); ESIMS *m/z* (rel. int.): 279.5 $[M + Na]^+$ (16), 257.5 $[M + H]^+$ (15), 256.6 $[M]^+$ (90), 254.5 (100), 239.5 (60), 221.5 (92), 203.5 (30).

3.9. Guaia-6α,7α-epoxy-4α,10α-diol (*4*)

Yellow oil; $[\alpha]_D^{20}$ −27.8 ($CHCl_3$; c 0.86); ^1H and ^{13}C NMR ($CDCl_3$) see Table 3; HREIMS *m/z* 254.1882 $[M]^+$ (Calcd. for $C_{15}H_{26}O_3$, 254.1882); EIMS 70 eV, *m/z* (rel. int.): 254 $[M]^+$ (20), 236 $[M - H_2O]^+$ (13), 221 (10), 218 (12), 151 (60), 123 (50), 109 (45), 107 (52), 87 (66), 81 (48), 71 (100), 55 (28).

3.10. Assay of antiproliferative activity against human prostate cancer cells

3.10.1. Cell culture

Human prostate cancer cell LNCaP and PC 3 were obtained from The American Type Culture Collection (Rockville, MD), and seeded in 100 mm dishes in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% CO_2 at 37 °C. Then, cells were maintained in serum-free RPMI 1640 medium for 24 h to deplete endogenous steroid hormones. Cells were next treated with tested compounds with mibolerone (Mib) in RPMI 1640 medium containing 5% charcoal-stripped serum. Mib was a synthetic androgen and not metabolized in cell culture. Mib and the compounds were dissolved in EtOH and DMSO, respectively.

3.10.2. MTT assay

MTT assay was used to measure cell inhibition of tested compounds in 96-well plates (Mosmann, 1983). The cells were treated with the test compounds for 96 h. After addition of MTT (10 μl/well, 5 mg/ml in phosphated-buffered saline), the plates were incubated for 2 h under 5% CO_2 at 37 °C. Then, the absorbance was determined at 570 nm.

3.10.3. Western blot analysis

LNCaP cells were cultured under the conditions described above and treated with **1** and **2** at various concentrations for 24 h in the presence or absence of 1 nm Mib. The subsequent processes were carried out according to the literature procedure (Yuan et al., 2004).

3.10.4. Transient transfection assay

The transient transfection assay was carried out according to the literature (Yuan et al., 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.01.013.

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